The Role of Blood-Brain Barrier Pathology in Post-Traumatic Epilepsy and its Co-Morbidities

By

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Dedication

I would like thank God for his Grace during my studies. He has been my source of strength and to him I give all glory.

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ABSTRACT

Traumatic brain injury (TBI) is becoming a global epidemic with an up-to-date figure putting its toll at 69 million people affected worldwide. In Canada, TBI accounts for 150,000 annual emergency room visits and about half-a-million people are living with a TBI-related disability. The more debilitating forms of TBI have also been associated with a higher risk of about 10-40% for developing post-traumatic epilepsy (PTE) as well as long-term cognitive impairments, neurodegenerative and neuropsychiatric diseases. There are currently no effective therapeutics for the prevention of PTE and associated co-morbidities. Accumulating evidence indicates that blood-brain barrier dysfunction (BBBD) is common following TBI and has a role in epileptogenesis. The goal of the present study was to test whether imaging BBB dysfunction following TBI can be used to predict the development of PTE and its associate co-morbidities.

Methods: We used a weight drop model of moderate traumatic brain injury in young adult rats. Rats were assessed for primary injury using a neurological score at baseline, 24, 48 hours and 1-week after injury. The magnitude of BBBD was assessed using a contrast-enhanced magnetic resonance imaging (CE-MRI) at 48 hours and 1-month time points. PTE was assessed using telemetric continuous electrographic recordings between 2-6 months after injury. Cognitive impairment was assessed using the Morris water maze test at 1 month after the trauma.

Results: CE-MRI confirmed BBBD 48hrs after injury in contrast to healthy controls. To this end, 6 rats (26%) developed PTE at 6-months post injury. Epileptic rats showed abnormal pattern of brain activity with increased occurrence of slow frequency events, termed “paroxysmal slow wave events” (PSWEs). Morris water maze confirmed a reduction in learning skills in animals after injury. The extent of BBBD at 48 hours was inversely related to performance at the Morris water maze, but not with the development of epilepsy at 6-months.

Conclusion: Post-traumatic epilepsy is fairly common following moderate traumatic brain injury. PSWEs may reflect an underlying neuronal hypersynchronous activity and may offer a novel non-invasive biomarker for neural injury and epileptogenesis. BBBD imaging may serve as a predicting biomarker for the development of cognitive impairment.

LIST OF ABBREVIATIONS USED

ABS	Acrylonitrile-butadiene-styrene
ANN	Artificial neural network
BBB	Blood-brain barrier
BBBD	Blood-brain barrier dysfunction
CDC	Centers for Disease Control and Prevention
CE-MRI	Contrast enhanced magnetic resonance imaging
FFT	Fast Fourier transform
GCS	Glassgow coma scale
ILAE	International League Against Epilepsy
MPF	Median power frequency
MMSE	Mini-Mental State Examination
MRI	Magnetic Resonance Imaging
NOR	Novel Object Recognition
NORT	Novel Object Recognition Test
NVU	Neurovascular Unit
PSWE	Paroxysmal Slow Wave Event
PTE	Post-Traumatic Epilepsy
TBI	Traumatic Brain Injury

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CHAPTER 1: INTRODUCTION

1.1 Traumatic Brain Injury

1.1.1 Background

Traumatic brain injury (TBI) has become a major focus of medical research in the 21st century, stemming from its socio-economic burden and rising toll globally (Polinder et al., 2013). In light of this, traumatic brain injury had been aptly described as 'a silent epidemic of our time' as society is oblivious of the enormity of its attendant complications (Chandel et al., 2016). Traumatic brain injury ensues when an external force such as a blow, jolt or bump impacts the head of an individual and this may result in disruption of brain function (Centers for Disease Control and Prevention) (Menon et al., 2010). The mechanisms of injury acquisition are largely heterogenous ranging from blast, penetrating, closed and crash injuries and equally disparate is the severity of clinical presentation and anatomic brain changes observed (Shlosberg et al., 2010). The location of the lesions following traumatic brain injury also ranges from focal to diffuse. The resulting force to the head causes a primary injury at the time of impact and this may result in cognitive, neurological and psychiatric sequelae developing months to years after the initial injury

1.1.2 Classification of Traumatic Brain Injury

Traumatic brain injury is traditionally categorized based on severity as mild, moderate or severe. This classification can be viewed as distinct entities of an increasing injury continuum. On one end of the spectrum are mild injuries constituting about 90% of all traumatic brain injuries in which TBI survivors are mostly asymptomatic and if symptomatic, residual effects are not common past the primary injury (Walker, 2011). On the opposite end of the spectrum are severe injuries which in contrast to mild injury, have

a poor prognosis and are often fatal (Salottolo et al., 2017). Flanking between these two spectra are moderate traumatic brain injuries, which are always symptomatic and associated with high risk of complications (Godoy, Rubiano, Rabinstein, Bullock, & Sahuquillo, 2016). About 10% of TBI patients will have a moderate to severe injury and close to 60 to 100% of these moderate to severe injury sufferers are disabled for the rest of their lives (Andriessen et al., 2011).

1.1.3 Epidemiology of Traumatic Brain Injury

Globally, around 69 million individuals experience TBI from all causes annually (Dewan et al., 2018). More concerning is the rate at which the number of individuals experiencing TBI is increasing as the world health organization (WHO) projects that TBI will be the third leading cause of disability and mortality by 2020 (Maas et al., 2008; Watanitanon et al., 2018). In fact, one study reported the case fatality as 1%, 21% and 40% for mild, moderate and severe TBIs, respectively (Andriessen et al., 2011).

Wood (2013) reports that the rising rate of TBI poses a huge financial expenditure of 302 million USD annually (Wood, 2013). In Canada, TBI accounts for about 150,000 annual emergency room visits and there are approximately half a million people living with a TBI-related morbidity (Hutchison et al., 2018). The burgeoning statistics in the literature underscores the toll of TBI socio-economic burden (Hutchison et al., 2018). TBI can be caused by falls, road traffic accidents, sport injuries and gunshot injuries. TBI caused by sports injuries has been described as the leading cause of disability in the first half of life whereas in the population of 65-year-olds and older, falls are the main cause of TBI (Watanitanon et al., 2018). Up to 25% of persons, above 65-years-old will experience a fall and this figure climbs to as high as 50% of the population in persons above 80-years. As

the population of persons above 65-years continues to expand faster than any demographic group in North America, more persons are at risk of suffering a TBI and its attendant complications. The higher the magnitude of the severity of TBI, the more likelihood of developing a TBI related complication.

1.1.4 Clinical Parameters for Assessing Severity of Injury

In classifying TBI to assess severity, explicating some injury severity indices may offer ample insights. One of the widely used earliest clinical measures for assessing the severity of a head injury is the use of the Glasgow Coma Scale (GCS). The GCS uses a combination of three variables, eye opening, motor response and verbal response to stratify patients into mild, moderate and severe injury. The ascribed score for each category is then summed up to arrive at the Glasgow Coma Scale for an individual and this is interpreted as 3-8, 9-12, and 13-15 for severe, moderate and mild head injuries, respectively (Teasdale et al., 2014). The GCS is a convenient, easy to administer clinical tool that finds great use for prognostication and assessment of clinical progress following head injuries (Teasdale et al., 2014). Similarly, an analogous neurological scoring method has been adapted for assessment of motor functions and anxiety in rodent models of traumatic brain injury (McAteer et al., 2016; Sweis et al., 2016). The neurological tests such as the open field, beam walk, and inverted mesh are carried out before and after induction of traumatic brain injury to assess severity of injury and to monitor progress at multiple time points (Tagge et al., 2018). Hence, the use of behavioural scoring in rodents, like the Glasgow Coma Scale is one of the commonly used indices for assessing injury severity in rodent models of traumatic brain injury. In addition to the use of the GCS, duration of consciousness and duration of post-traumatic amnesia are other clinical tools that can be used to assess

severity of traumatic brain injury. The longer the duration of loss of consciousness and post-traumatic amnesia, the more the severity of injury (Greenwald at al., 2003).

Mortality rate is another measure for assessing injury severity following a traumatic brain injury (Kharatishvili et al., 2006). McIntyre (2012) reports a mortality of 12.3%, 34.3% and 65.3% for mild, moderate and severe injury (McIntyre et al. 2013). However, Andriessen (2011) reports the case fatality rate following moderate to severe TBI to be 21% and 46% respectively (Andriessen et al., 2011; Zafonte et al., 1997)

1.1.5 Imaging Techniques Employed in Assessing Severity of TBI

The twenty-first century has witnessed an unprecedented growth in the use of radiological diagnostic techniques like magnetic resonance imaging (MRI) and computed tomography for visualization and assessment of structural brain pathologies (Badaut et al., 2019; Levine, 2006). The use of neurological imaging has gained ground as the standard of care for investigating head injury patients (Gerber et al., 2004) and it has also been shown to correlate with severity of head injury (Badaut et al., 2019; Lowenstein, 2009). Newer capabilities of these expanding anatomical imaging techniques continue to develop as it is increasingly being used to view brain hemodynamic changes (Metting et al., 2007). This can reveal varying degrees of neuropathologies such as contusions, subarachnoid hemorrhage and subdural hemorrhage. It can be conducted at an acute time point following head injuries as well as delayed time points to assess the injured brain. Using MRI, whole and regional brain analysis can be carried out (Bigler, 2013). Most focal traumatic brain injuries are known to affect the temporal and frontal cortex (Chew et al., 2014), as imaging based mapping reveals anatomic brain changes in these regions (Chew et al., 2014). The functional correlates of the acute brain changes observed on MRI and the development of

long-term complications are still in the early stage. Hence, the MRI is a technique that can be employed in human and animal models to view the acute pathological substrates that results in chronic complications (Immonen et al., 2009; Metting et al., 2007). In the light of this, the MRI may be useful to predict long-term neuro-cognitive complications that follow head injury (Haghbayan et al., 2016).

1.2 Complications of Traumatic Brain Injury

Many patients who experience a moderate to severe TBI are prone to developing long-term neurological and psychiatric complications months to years after the primary insult to the brain (Tomkins et al., 2011). Studies in humans and animals have revealed that higher order injuries increases the likelihood of developing complications such as cognitive disorders, neurodegenerative diseases and post-traumatic epilepsy (Kharatishvili et al., 2006; Pitkänen et al., 2009; Tajiri et al., 2013; Wijayatilakea et al., 2015). Here, the enumerated TBI complications will be discussed in more details.

1.2.1 Complications of Traumatic Brain Injury: Cognitive Impairment

The ancillary role that traumatic brain injury plays as a risk factor for neurodegenerative diseases such as Alzheimer's disease (Johnson et al., 2010; Sivanandam et al., 2012; Uryu et al., 2007) and Parkinson's disease (Chase, 2015; Wong & Hazrati, 2013) has been well acknowledged by several studies: such studies have placed tau phosphorylation, Aβ deposition, synaptic pruning and cell death on the map of the pathological features common to TBI and these diseases. A commonality shared by both TBI and neurodegenerative disease is the inflammatory response which may mediate the secondary process that culminates as cognitive impairment following TBI (Wilcock, 2014).

Indeed, accumulating evidence indicates that the risk of developing cognitive impairment as well as dementia more than doubles proportionally with increasing severity of traumatic brain injury (Gottlieb, 2006; Vincent et al., 2014) with a greater likelihood of developing cognitive impairment in moderate to severe TBI as opposed to mild injury (Wang & Li, 2016). Interestingly, similar to posttraumatic epilepsy where most seizures are of focal-onset arising mostly from the temporal and frontal cortex, tau and amyloid plaques have also been shown to be deposited in the frontal and temporal cortex (Jagust, 2012).

Evidence exists to that a similar cognitive decline following TBI in human subjects has also been recapitulated and observed using animal models of traumatic brain injury. The Morris water maze (MWM) is commonly employed to test for learning and memory (Karl et al., 2012) and this test can be used to assess immediate recall and remote memory in rodents, quite analogous to the Mini-Mental State Examination (MMSE) employed in humans to test for cognitive impairment. Rodents go through a period of learning at which time they are trained to locate an obscure platform in a hidden pool. After these learning trials, immediate and remote spatial memory are assessed by removing the platform (Vorhees & Williams, 2006). Also, other studies have employed the novel object recognition test for assessing learning and memory in rodents (Lueptow, 2017; Matsumoto et al., 2014). Consequently, these cognitive tests have been used following traumatic brain injury in rodent models to assess for learning and memory deficits (Tucker et al., 2018). Even though TBI has been established as a common phenomenon that can result in secondary complications such as neurodegenerative diseases and other neural dysfunctions such as posttraumatic epilepsy and slow wave events, an emerging area of interest by researchers is to decipher the mechanism(s) by which TBI results in delayed complications.

Understanding the mechanism(s) involved would serve as potential therapeutic avenues for targeted drug development and could also ultimately lead to the development of a biomarker to detect TBI survivors who have high risks of developing PTE and its co-morbidities.

1.2.2 Complication of Traumatic Brain Injury: Post-Traumatic Epilepsy

1.2.2.1 Definition of Post-Traumatic Epilepsy

Post-traumatic epilepsy (PTE) is a common complication of traumatic brain injury. The International League Against Epilepsy defines epilepsy as the occurrence of at least two unprovoked seizures occurring more than 24 hrs apart or "the occurrence of one reflex seizure and a probability of further seizures similar to the general recurrence risks after two unprovoked seizures" (ILAE 2014). Such individuals have an enduring propensity to initiate and propagate epileptic seizures and these may manifest as motor, sensory and psychic phenomena (Fisher et al., 2005). Many neurologic diseases are almost invariably accompanied by some degree of impairment of transmission of impulses. Therefore, it is not surprising that such disorders frequently present with a non-pathognomonic finding of seizure, and, as the severity of injury increases, the risk of developing seizure also increases (Ahlbom et al., 2015; Annengers & Rocca, 1998)

1.2.2.2 Epidemiology of Post-Traumatic Epilepsy

Recent studies reveal that PTE rates range between 2-50% (Tomkins et al., 2011; Uski et al., 2018); some studies suggest that PTE is the most prevalent cause of acquired epilepsy with an incidence of 20% (Reid et al., 2016; Shultz et al., 2013). The risk of developing PTE for moderate to severe TBI is between 20-50% (Klein et al., 2018; Lowenstein, 2009; Pitkänen et al., 2014). The likelihood of developing PTE is dependent

on the age at insult: with children being more susceptible compared to adults (Webster et al., 2017). Accumulating evidence from neuroimaging reveals that higher-order injury in the moderate to severe spectrum are associated with structural brain changes like intracranial hemorrhage, subarachnoid hemorrhage, brain contusions and subdural hemorrhage and these changes ultimately increases the risk for developing PTE (Xu et al., 2017)

1.2.2.3 Classification of Post-Traumatic Seizures

Posttraumatic seizures may be further classified according to the time of seizure occurrence: immediate for a seizure occurring within the first 24 hrs post injury, early for a seizure occurring between 24 hrs and 1 week, and late - for seizures that arise after the 1 week post injury induction (Ritter et al., 2016). The immediate and early seizure subtypes are broadly classified as acute seizure while recurrent late seizures constitute PTE. An interesting distinct point is that unlike acute seizures that are manageable with the use of the widely available antiepileptic medications, the same drugs are ineffective in preventing PTE (Chang & Lowenstein, 2003; Wilson et al., 2018). PTE is often also pharmacoresistant (Kharatishvili et al., 2006; Klein et al., 2018; Webster et al., 2017) and associated with significant morbidity and neuropsychiatric complications (Wijayatilakea et al., 2015). This highlights the need for research into the detailed mechanisms underlying PTE (Garga & Lowenstein, 2006). But first, there is a need to better understand seizure phenomenology.

Neurons have the capability of generating membrane currents and these currents can be detected by electrodes either placed over different regions of the cerebral cortex or over the surface of the scalp; aptly described as electrocorticography or electroencephalography, respectively. The measured recording at any given region of the

brain mirrors the summation of the many overlying and superimposed field potentials of the surrounding neuronal cells. This technique gives insight into the spatiotemporal workings of neurons and has been the gold-standard for detecting brain epileptiform activity (Elger & Hoppe, 2018). Furthermore, to confirm the clinical significance of changes observed in brain activity, continuous video recordings may be used for seizure characterization (Elger & Hoppe, 2018). The utility of seizure detection techniques has illuminated our understanding of seizure classification based on the origin of abnormal excessive hypersynchronous neuronal discharge and semiology observed during seizure. This means that the clinical presentation of seizures is dependent on the spatial origin of epileptic discharges and the extent to which such discharges are propagated in the brain. Hence, the clinical presentation of seizures is not stereotypic, however, seizure symptoms may be stereotypic if an epileptic discharge originates from the same focus.

1.2.2.4 Clinical Manifestation of Seizures

In 2017, the International League Against Epilepsy (ILAE) revised the 1989 criteria to reflect the recent advances in epilepsy research. The criteria for the classification of seizures were separated into three categories: focal-onset, generalized-onset seizure and unknown. In focal onset seizure, hypersynchronous neuronal firing is limited to a brain hemisphere and usually present with a seizure on one side of the body. The ILAE further categorized focal-onset seizures into focal aware seizures, focal impaired awareness seizures, focal motor seizures, focal non-motor seizures, and focal to bilateral tonic-clonic seizures (Scheffer et al., 2018). Likewise, for generalized onset epilepsy, the excessive and abnormal neuronal firing involves both hemispheres of the brain. This seizure sub-type may manifest as absence, myoclonic, atonic, tonic, and tonic-clonic seizures (Scheffer et

al., 2018). About 60% of epilepsies are of focal-onset and a good number of these epileptic discharges arise from the temporal and frontal lobe. Generally, an injury that affects the same focus of the brain in individuals manifests with stereotypic semiology. The symptoms are largely heterogenous between people when different brain regions are injured. To illustrate, frontal lobe seizures manifest with behavioural arrest, complex automatisms, repetitive semiology, duration less than 30 seconds, and tend to occur during sleep (Andriessen et al., 2011; Gupta et al., 2014; Reid et al., 2016). In the same vein, temporal lobe seizures manifests with aura, behavioural arrest, memory impairments, automatisms and autonomic dysregulation (Berkovic et al., 1996; Sloviter, 2005). Temporal lobe and frontal lobe epilepsy are the most common type of focal seizures and these epilepsy types constitute 57% and 35%, respectively (Gupta et al., 2014) while parietal lobe and occipital lobe epilepsy constitutes only 3% each of focal epilepsy (Gupta et al., 2014). By extension, accumulating evidence has shown that some models of TBI produce a signature injury to various regions of the brain and this is an invaluable tool for studying epileptogenesis (Kharatishvili et al., 2006). Most of these TBI models report that the seizures are of focal-onset or secondarily generalized seizures (Lamar et al., 2014; Pitkänen & Immonen, 2014). More insights from the characterization of the seizure events over time in an animal model of TBI revealed that seizure frequency and duration increases with severity of TBI and time post injury induction (Reid et al., 2016).

Other abnormal patterns of brain activity based on slow frequency events and power spectrum dynamics are proposed to reflect an underlying excessive abnormal neuronal hyperexcitability (Milikovsky et al., accepted). A novel yet emerging concept is the paroxysmal slow wave events which are episodic events defined by median power

frequency less than 5 Hz lasting for 10 seconds (Milikovsky et al., accepted). These events may serve as a specific biomarker of an epileptic brain. Transient dynamics in baseline neuronal firing have been observed in an ageing brain as well as disorders that result in accelerated brain ageing like Alzheimer disease and traumatic brain injury. And since TBI is a recognized risk factor for posttraumatic epilepsy and other neurodegenerative diseases, slow wave events might reflect an underlying epilepsy. In addition to slow wave events, previous studies have shown that power spectrum changes could potentially be significant for detecting epilepsy and epileptogenesis (Milikovsky et al., 2017). Indeed, electroencephalogram (EEG) background slowing has been reported to be associated with epileptic seizures and traumatic brain injury: theta and delta bandwidth is increased in TBI and epileptic patients, whereas, alpha and gamma bandwidth is increased in healthy controls compared to the lesioned brain (Kilias et al., 2018; Milikovsky et al., 2017; Tomkins et al., 2011). In fact, evidence exists to suggest diffuse background EEG transition also occurs following focal seizures (Perucca et al., 2013). Therefore, power spectrum frequency bandwidth and slow wave events are potential non-invasive biomarkers which may reflect an underlying neuronal hyperexcitability.

1.3 The Problems

Some of the current problems with TBI research include little understanding of the mechanism(s) by which TBI culminate in PTE and also the lack of biomarkers that can be employed to adequately predict TBI patients at risk of developing epilepsy. Some propositions in favour of the possible mechanism(s) and evolving biomarker research have been reported and will be briefly described here.

Briefly, inflammation is a potential neurobiological mechanism which has been prominently purported in the literature to play a role in igniting the cascading pathway which culminates in epilepsy. Research insight has now revealed that the brain, once thought to be an immunologically deprived organ, contains cells capable of eliciting inflammatory response (Stewart et al., 1997). Similarly, a specific neuroinflammatory chemokine (Il-6) was found to be up-regulated by astrocytes following traumatic brain injury (Levy et al., 2015). The evidence for the mechanistic link by which TBI results in up-regulation of the chemokines and epilepsy has been reviewed recently (Friedman, 2011; Milikovsky et al., 2017) and will be succinctly outlined here. The epileptogenic process is presumably initiated by the extravasation of albumin, the most abundant serum protein, into the brain macroenvironment after traumatic brain injury (Friedman, 2011;Tomkins et al., 2007). This extravasated protein subsequently binds to a transforming growth factor β (TGF-β) receptor expressed on astrocytes thereby triggering downstream phosphorylation cascades (Cacheaux et al., 2009; Ivens et al., 2007). In line with this inflammatory astrocytic response; neuronal hyperexcitability, synaptic pruning and neuronal loss may ensue manifesting as PTE and other neurodegenerative diseases (Friedman, 2011). Publications by Bar-Klein et al. (2014) and Weissberg et al. (2015) serve to give credence to the above proposition as losartan and IPW (TGF-β blocker) were employed successfully to block TGF-β signaling (Bar-Klein et al., 2014; Weissberg et al., 2015). Although, this potential therapeutic target seem plausible, an expert review by Friedman et al (2014) revealed that administering losartan prophylactically to TBI survivors would be tantamount to an exercise in futility as it will mean treating some subjects unnecessarily (Friedman et al., 2014). Hence, the search for a biomarker that is capable of predicting TBI survivors at

a high risk of developing PTE has been a centerpiece subject for researchers (Milikovsky, Weissberg, et al., 2017; Pitkänen et al., 2016).

The Pitkanen et al. (2019) treatise on epilepsy biomarkers aptly describes a biomarker as a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions"(Pitkänen et al., 2019). A biomarker would hold much promise in facilitating the identification of TBI survivors who are at high risk of developing epilepsy, thereby making it a potential therapeutic preventive avenue without having to wait until epilepsy develops (Engel, 2019).The evidence currently available suggests that posttraumatic epilepsy develops weeks to months following the primary insult to the brain with an even shorter latency in moderate to severe head injuries (Pitkänen et al., 2014;Tomkins et al., 2011). Recent research has provided tremendous insights to several putative biomarkers such as electrocorticographic changes (Kim et al., 2018; Milikovsky et al., 2017), neuroimaging (Bar-Klein et al., 2017; Veksler et al., 2014), optical (Szu, 2018), genetic (Cotter et al., 2017), serum (Dadas et al., 2018), and molecular changes (Pitkänen et al., 2016). Many unprecedented strides have been achieved since the inception of the search for a biomarker, however, the most promising results have emerged from imaging studies (Pitkänen et al., 2014).

1.4 The Blood-Brain Barrier

1.4.1 Anatomy and Physiology of the Blood-Brain Barrier

The BBB is a structural and functional framework of the neurovascular unit (Milikovsky et al., 2017), housing a convoluted web of neural matter and pericytes that

serve as the cornerstone for regulating the brain homeostasis (Abbott et al., 2010), with closely interacting astrocytes extending via their foot processes (Abbott & Friedman, 2012) (Figure 1). The delicate toil by which the BBB maintains the brain microenvironment is meticulously executed by the triumvirate units of the endothelial cells, the endothelial cell basement membrane and tight junctions (Gürsoy-özdemir & Tas, 2017). Within the conceptualized anatomic framework of the BBB are functionally distinct carrier proteins mediating transport of glucose, amino acids, ions and macromolecules such as protein and peptides. Hence, in the physiological state, the BBB functions via a non-mutually exclusive mechanism to allow gaseous exchange, movement of nutrients and wastes and other selective particulate matter (Gürsoy-özdemir & Tas, 2017). However, an alteration in the brain milieu may ensue following perturbation of the BBB as can occur following traumatic brain injury and other neurological diseases (Tomkins et al., 2011). Hence, there is burgeoning evidence that BBB disruption is a critical nodal point affected following traumatic brain injury (Abbott & Friedman, 2012; Bar-Klein et al., 2017; Milikovsky et al., 2017), but of course like any theory, it is not without unknowns. One such question is which neurobiological mechanism is presumably activated following the disruption of the vascular-neuronal interface in PTE (Friedman, 2011).

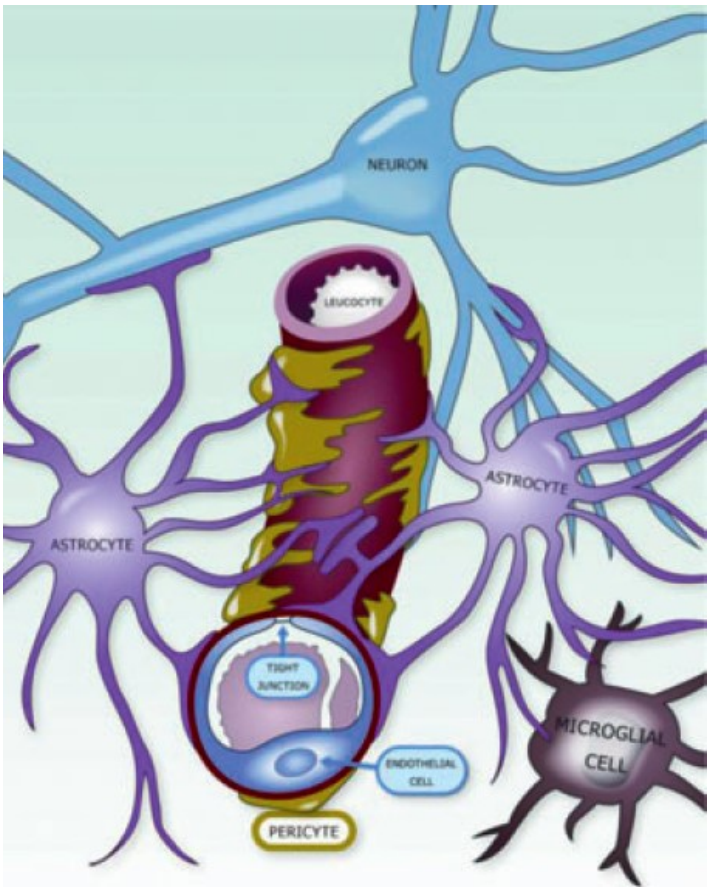

Figure 1. Schematic drawing of the Neurovascular Unit. The figure illustrates the anatomic and physiologic relationship between astrocytes, microglia, pericytes and endothelial cells. (Abbott & Friedman, 2012).

1.4.2 The Role of Blood-Brain Barrier in PTE and its Co-Morbidities

Accumulating research has offered insight and has revealed that the BBB may be the lynchpin linking TBI with long term neurocognitive sequela (Figure 2A) (Dadas & Janigro, 2019;Tomkins et al., 2008). Not only has the opening of the structural framework been described following TBI, but also mounting data exists that a similar BBB disruption occurs following neurological disease such as meningitis, multiple sclerosis, stroke, and Alzheimer's disease (Figure 2B) (Chassidim et al., 2015; Serlin et al., 2019; Veksler et al., 2014; Zenaro et al., 2017). Expectedly, animal models and human data have revealed an early disruption of this brain vascular-neuronal interface (Shlosberg et al., 2010; Tagge et

al., 2018;Tomkins et al., 2011) with natural recovery occurring within a week, although, BBB opening may be persistent beyond a week in moderate to severe TBI (Wang & Li, 2016).

As earlier described, neuroimaging has great potential in being employed as a non-invasive biomarker. This imaging technique entails the use of dynamic contrast- enhanced magnetic resonance imaging (DCE-MRI) to measure the temporal and spatial permeability of the blood-brain barrier (Bar-Klein et al., 2017; Levy et al., 2015; Veksler et al., 2014). With this technique, a gadolinium-based contrast agent that does not cross the BBB in an healthy brain is injected into a peripheral vein after which a BBB permeability map is acquired (Veksler et al., 2014; Weissberg et al., 2014). Tomkins et al. (2011) observed BBB opening with increasing severity of the TBI. Later publications in rodent models and human patients are in keeping with post-injury BBB opening (Bar-Klein et al., 2017; Dadas & Janigro, 2019; Weissberg et al., 2014). This raises a pertinent question, could BBBD be used to predict TBI survivors with a high risk of developing long term complications like PTE and cognitive impairment?

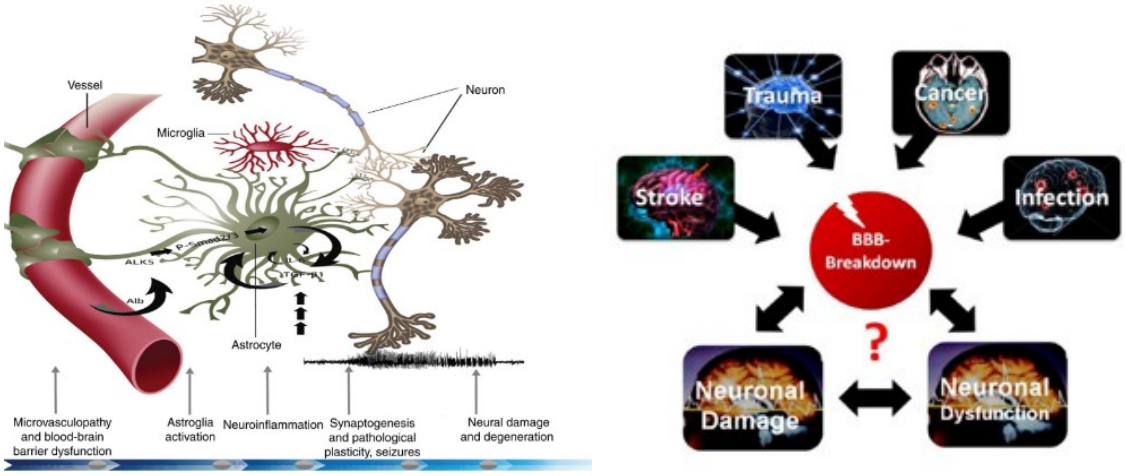

Figure 2. Schematic drawing of a disrupted blood-brain barrier following TBI and its role in PTE and other neurological diseases (Adapted from Bar-Klein et al., 2014)

(A) Mechanism underlying PTE and neurodegeneration after TBI (B) BBBD is a common final pathway in PTE and other neurological diseases.

1.5 Objectives and Hypothesis

My main objective is to evaluate if blood-brain barrier dysfunction (BBBD) predicts posttraumatic epilepsy in a rat moderate traumatic brain injury model. In addition to the above objective, the predictive ability of BBBD in the development of cognitive impairment will also be evaluated. Finally, potential electrocorticographic biomarkers of epilepsy will also be assessed. Hence, I hypothesize that BBBD is a predictive biomarker for the development of post-traumatic epilepsy and its co-morbidities.

1.6 Goals and Rationale

While the nodal point that TBI sub-serves in the knotty loop that results in PTE and other neurodegenerative diseases, has been established, the mechanism(s) by which TBI is linked to these long-term sequelae continues to evolve. Accumulating evidence has revealed the BBB as a plausible link between TBI and the development of PTE and its co-morbidities. In fact, the risk of developing PTE and other neurodegenerative diseases increases with severity of TBI and the extent of BBB opening (Dadas & Janigro, 2019; Reid et al., 2016;Tomkins et al., 2008; Wang & Li, 2016). Even worse is the fact that seizure resulting from traumatic brain injury are pharmacoresistant (Klein et al., 2018; Webster et al., 2017) and there are currently no effective therapeutics for the prevention of PTE and associated co-morbidities (Saletti et al., 2019; Wilson et al., 2018). As can be recognized, a search for a biomarker becomes imperative as there is currently no biomarker

capable of identifying TBI survivors at risk of developing these long-term complications (Engel, 2019; Pitkänen et al., 2019; Pitkänen & Immonen, 2014). Many biomarker advances have been made in recent years (Dadas et al., 2018; Milikovsky, Weissberg, et al., 2017; Pitkänen et al., 2016), nonetheless, the use of neuroimaging has been the most revealing (Pitkänen & Immonen, 2014). Hence, I hypothesize that the use of neuroimaging (a non-invasive biomarker) to assess blood-brain barrier disruption following traumatic brain injury predicts epilepsy and its co-morbidities.

Here, we used a weight drop model of moderate TBI to study if BBB dysfunction following induction of injury can be used to foretell animals that will develop epilepsy and its co-morbidities. The weight drop model employed recapitulates the common mechanisms of injury acquisition in humans such as sports injuries, falls and road traffic accidents. It also has been shown to be a good model to study posttraumatic epilepsy (Hou et al., 2017; Pitkänen et al., 2014).The model was established in our laboratory and was verified to be moderate TBI (Hou et al., 2017) based on mortality rate, neurological status, delayed recovery post-injury induction and gross pathology on the brain.

CHAPTER 2: METHODS

2.1 Experimental Animals and Husbandry

Before the experiments were performed, consent was obtained from the Dalhousie University animal care committee and experiments were conducted in compliance with the Canadian Council for Animal Care guidance. This experiment used 10-week old male Sprague-Dawley rats (procured from the Charles River Farms) weighing 320 – 430g. 8 weeks old rats were ordered and allowed to habituate to the animal care facility for 1 week and to eliminate the effect of travel stress. During this time of habituation, animals were handled, and daily weights were obtained and noted. At 9 weeks, animals were trained on the beam (one of the tasks used to assess neurological deficits) for three consecutive days preparatory for the experiments proper. From the time the animals arrived at the facility to their final end points, the animals were housed in a reverse light-dark cycle between the hours of 9 am to 9 pm. Food and water were administered to the animals ad-libitum and the animals were randomly housed in groups of two. Animals were also blindly allotted to control and TBI groups. A group of 39 rats was used to establish the novel weight drop moderate traumatic brain injury model (elucidated further below). After validating the weight drop model, a cohort of 68 TBI and 22 control rats were used in assessing the role of blood-brain barrier in the development of long-term neurocognitive sequela.

2.2 Establishing the Moderate Weight Drop Model

Thirty-nine rats were used to establish the moderate weight drop model (Marmarou, 1994) to determine which weight and height recapitulates the neuro-behavioural symptoms expected for a moderate closed head injury. 450- and 500-gram weights and a height of 1.2m were used for TBI induction. Baseline neurological scores and weights for each

animal were collected. Before injury induction, animals were anesthetized using 3% isoflurane at 2L of oxygen for 2 minutes. To assess the depth of anesthesia, animals were toe pinched and an additional 3% isoflurane was administered for 1 minute. Animals who responded to the toe pinch received an extra minute of isoflurane. The animal was then placed on a foil overlaying the open top of a rectangular plexiglass box with a foam pad at the base (Figure 3). The foil is in place to cause rotational movement with the fall after the hit. Once the animal is placed on the foil, a cylindrical disk is then placed over the scalp, midpoint between both ears and the weight impactor was released from a height of 1.2 m which falls freely to hit the disk placed on the rat skull. Following induction of TBI, animals were quickly transferred to a recovery box for 20 minutes and were filmed throughout the process of recovery. After 20 minutes of recovery from the TBI, neurological severity scoring was employed for assessing primary injury using three behavioural tests. The tests were repeated at 24, 48 hours and 1-week post injury induction to monitor neurological changes, if any. The procedure was carried out during the dark phase of the light-dark cycle.

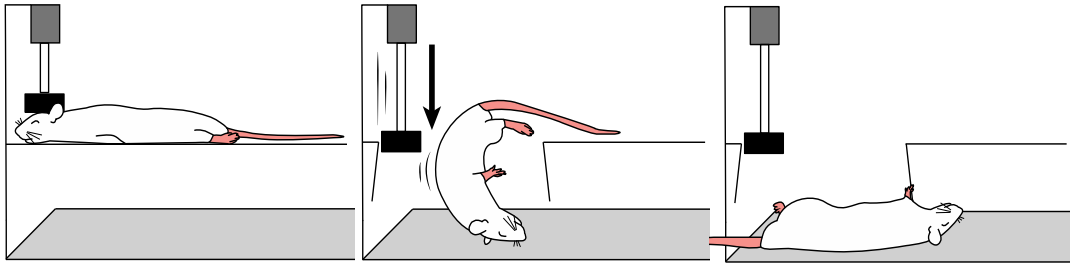

Figure 3. A schematic of the TBI apparatus showing animal on the foil, rotational movement, and foam pad.

2.3 Experimental Paradigm

After validating the moderate TBI model using a 450-gram weight and a height of 1.2m, we set forth to use this weight in subsequent experiments. Refer to Table 1 for experimental timeline.

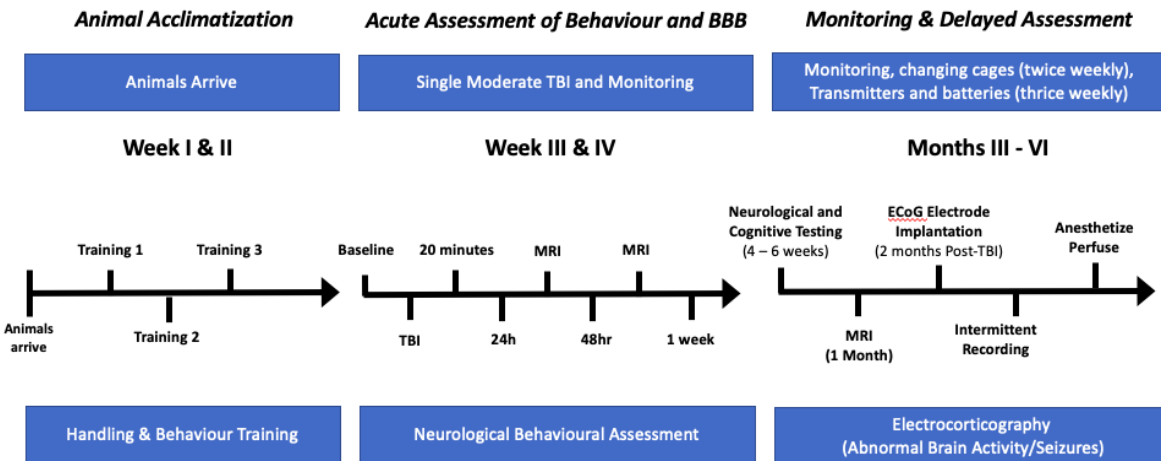

Table 1. Schematic of the experimental timeline from arrival to the final end point.

2.4 Neurological Testing

Three tasks (open field, beam walk, and inverted wire mesh) were used for neurological testing. Following each test, animals were ascribed a score for each component of the tasks and a composite score were then obtained by summing scores from each of the three tests to arrive at the neurological severity score (Tagge et al., 2018) (Table 2).

Open field: As previously described by Kuniishi et al. (2017), the open field apparatus is a square arena 23x23 inches with walls 23 inches high made from black plexiglass (Kuniishi et al., 2017). The tasks were filmed with a video camera and tracked using Ethovision (Noldus) software which uses the contrast between the box and the animal's body to capture the image of the animal. The center point of the body was used

as the reference point when capturing body image. The arena was divided into peripheral and center zones. At commencement of the tasks, a score of 1 was ascribed for every corner the animal visits up to a maximum of 4 if the animal visited all four corners of the box with each test lasting 45 seconds (Figure 4A). In between animal trials, the box was wiped clean with hydrogen peroxide.

Beam walk: The beam walk apparatus consists of a horizontal beam with a darkened square box, large enough for the animal to enter, situated on one end of the beam. Rats were placed on the opposite end of the beam from the box and 1 minute is given to each animal to cross the beam into the box (Figure 4B). The beam walk is scored based on the distance covered on the beam with a maximum score ascribed when the animal enters into the box or its position when one minute has elapsed (Sweis et al., 2016). Scores range from 0 for an immobile rat to 4 for animals who entered into the box. Any fall from the beam during testing was noted.

Wire Mesh: This is a square-shaped apparatus made of wire mesh contained in a 14 by 14-inch frame. The mesh is 0.5 by 0.5-inch squares. The rodent is placed on the mesh and it is carefully inverted. It is inverted for a maximum of 5 seconds. If the rodent falls from the mesh within the 5 seconds, the time is noted. The longer the duration the rat remains on the mesh, the higher the score ascribed with a maximum possible ascribed score being 4 (Figure 4C).

Figure 4. Neurological testing apparatus (A) Open field test. Captured image of the rat in one corner. (B) Beam walk apparatus. A rat captured moving over the beam into the box. (C) Mesh apparatus. A rat captured on the inverted wire.

A	Test 1: Open Field (45 seconds)	4 Visits 4 corners	3 Visits 3 corners	2 Visits 2 corners	1 Visits 1 corners	0 Does not visit any corners
B	Test 2: Inverted Wire Mesh (5 seconds)	4 Climbs on top of mesh	3 Hangs on for ≤ 5s	2 Hangs on for ≤ 3s	1 Hangs on for ≤ 1s	0 Paralysis
C	Test 3: Beam Walk (60 seconds)	4 Crosses full length	3 Crosses ¾ length	2 Crosses ½ length	1 Crosses ¼ length	0 Does not move from start

Table 2. Neurological scoring (A) Open field test. Scored from 0 to 4 based on the corners visited within 45 seconds. (B) Wire mesh. Scored from 0 to 4 based on how long the rat clings to the mesh within 5 seconds. (C) Beam walk. Scored from 0 to 4 based on the distance covered before entering the darkened box within 60 seconds.

2.5 Magnetic Resonance Imaging

Magnetic resonance imaging scans were carried out at the Biomedical Translational Imaging Centre (Biotic) using a 3 Tesla machine. The imaging protocol to assess BBB permeability included (1) T2-weighted fast spin echo (FSE) scan (TR, 2.5s; TE, 68ms), echo train length,16; echo spacing, 8.5ms; 46 averages, axial slices, 1.2 mm thick, 14 slices; approximately 200 μm in-plane resolution (2) Dynamic contrast-enhanced MRI (DCE-MRI) scans (TR, 1min; TE, 1 min), flip, 20; 20 averages; axial slices, 1.2 mm thick; 14

slices, approximately350 μm in-plane resolution; 9 volumes; 1 pre-contrast; and 8 post-contrast. Time resolution was approximately 3 min per volume. The contrast agent used was an intravenous (iv) injection of 0.4 ml of Multihance. (3) High resolution 3D anatomical scan – balanced steady state free precession (BSSFP) (TR, 8ms; TE, 4ms); 1 offline average; 4 frequencies; 20 dummy scans; 10s segment delay; flip, 60; resolution 0.25 mm x 025 mm x 0.3 mm. Using the above imaging protocol, subgroups of rats were scanned at 48 hours and 1 month respectively.

2.5.1 MRI Data Analysis

Data collected using DCE-MRI was analyzed for BBB as described (Chassidim et al., 2015; Veksler et al., 2014). Briefly, the protocol involved using an in-house MatLab script to analyze dynamic changes in signal following the injection of gadolinium-based contrast agent-Multihance. Linear fit was used, and slope results were calculated. Cumulative frequency analysis of slope value was performed and the 85^{th} percentile of control brains (N = 9) was used as the threshold for a "pathologically high" permeability. The percentage of brain voxels with a supra-threshold "pathological" value was calculated for each brain.

2.6 Cognitive Testing

Cognitive impairment was tested using the novel object recognition test and Morris water maze test. First, the novel object recognition test (NORT) was performed using a 23 x 23 inch square darkened box as previously described (Lueptow, 2017). This test was performed at 1-month post injury induction. The NORT involved three consecutive sessions in which each rat went through a period of 2 minutes habituation, followed by 5

minutes of training, and finally 3 minutes of testing. In the habituation phase, the animals were placed inside the open field arena with no object and allowed to traverse freely. Then the animals were removed, and two identical objects were placed in opposite quadrants about 5 cm apart. The animals were returned to the open field for 5 minutes in the training phase. Animals were returned to their home cages for 5 minutes, during which one of the identical objects was replaced with a novel object. Following this, animals were returned to the open field for the 3-minute test phase. A camera connected to the Ethovision software was placed overhead to track the exploratory movements of the rats during the sessions and a nose-point detection was used as the reference point for image tracking (Figure 5A). The percentage of time each animal spent exploring the familiar and novel objects was then calculated using the Ethovision software.

Cognitive testing was also performed using the Morris water maze (Figure 5B) test between 2-4 months post injury induction. As previously described, the Morris water maze assesses learning and spatial memory (Zhou et al., 2016). A hidden platform obscured in a circular pool filled with water at room temperature of dimensions: diameter, 210 cm; height, 51 cm was used for the task. Four coloured shapes serving as visual cues were placed on the walls around the circular tank at four different quadrants. During the training and probe sessions, the platform was left at the same place inside the pool of water. Prior to the initial probe trial, 6 training trials each starting from different spatial locations were performed for each rat, following which a single probe trial was performed for each rat. A duration of 60 seconds was allotted for training trials and an additional 30 seconds is allotted if the rats finds the platform, otherwise, rats unable to locate the platform in 60 seconds were guided to the platform and allowed to habituate for 30 seconds. For the single

probe trial, a duration of 60 seconds was allotted for each rat without the platform in the circular pool. Following these sessions, 3 training trials are then performed again with the platform in the same location as previously described. To assess remote memory, a repeat probe trial is performed on the rats 24 hours after the last training sessions. The training and probe trials were all recorded with the aid of an overhead camera connected to Ethovison software. The parameters of interest during the probe trials were latency and total distance to the platform arena as well as the cumulative duration spent in the platform arena.

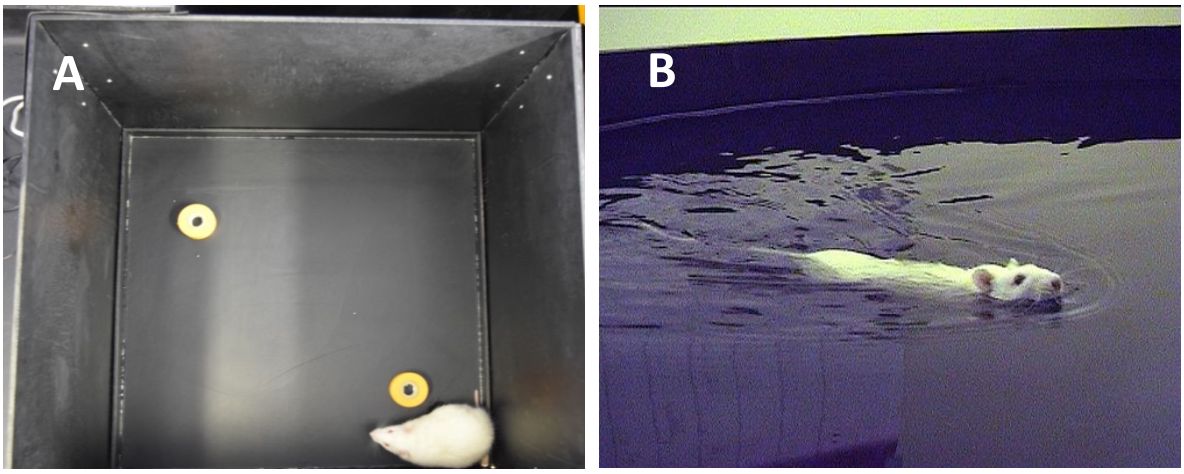

Figure 5. Cognitive testing apparatus (A) Novel object recognition test. Captured image of the rat with identical familiar objects. (B) Morris water maze test. An image of a rat captured while swimming during the probe trial.

2.6.1 Morris Water Maze and Novel Object Recognition Analysis using Ethovision

The Ethovision video tracking software was used in analyzing the Morris water maze (MWM) and novel object recognition tests results. The parameters of interest were latency to the novel object and distance moved in the open field for the novel object recognition tests while mean distance to platform and cumulative duration spent in the platform arena were the outputs variables chosen for MWM (Vorhees & Williams, 2006)

for immediate and 24 hour probe trials. For both cognitive tests described, the Mann-Whitney test was used to compare the control and TBI groups.

2.7 Electrode Implantation Surgery

Starting from 2 months post injury induction, rats were implanted with five epidural electrodes made of stainless steel without breeching the dura. Briefly, the procedure entailed anesthetizing the animals using 3.5% isoflurane at 2L of oxygen for 2 minutes for induction and 2% isoflurane at 2L of oxygen for maintenance. When the animal was deeply anesthetized, a local anesthetic agent, bupivacaine was injected around the incision site at 8mg/kg. Long-acting subcutaneous buprenorphine (sustain release) at 1.2 mg/kg and intraperitoneal ketoprofen at 5 mg/kg were also administered for analgesia. Animals were placed in a stereotaxic frame; hair shaved; skin decontaminated using hibitane, alcohol and betadine; sterile drape placed over the scalp; and a midline incision made over the scalp to expose the skull. The subcutaneous tissue was cleaned away from the skull using a spatula to reveal the suture lines. Taking bregma as the reference point, four 0.8 mm diameter holes were drilled 2 mm and 6 mm on the right and left skull posterior to bregma and 3 mm laterally. The fifth hole, for the ground electrode was drilled 2 mm posterior and lateral to lambda. The stainless screws on one end of the Teflon wire electrode were inserted into the drilled holes. The other end of the electrode was attached to a plug made of acrylonitrile-Butadiene-Styrene (ABS). Dental acrylic and sutures were used to secure the electrodes around the headpiece. Following surgery, animals were housed singly in their home cages and were allowed a week post-surgery to recover after which a battery-

powered wireless transmitter (EMKA) was placed over the electrodes to monitor the brain electrical activities.

2.8 Video-ECoG Recording

Brain signals emanating from the affixed transmitters were detected by two receivers (1KH sampling rate) placed 2 m above the rat cages and these signals are transmitted to an EMKA computer and are filtered. Continuous brain activities were recorded for a minimum of two weeks for all animals. Animal behaviour were also continuously recorded using a video camera (EMKA) which was positioned above the rat's cages and are conned to the Emka computer via cables. The built-in infra-red lens of the video camera as well as having a wide-angle lens also allowed for day and night recording.

2.8.1 Video-ECoG Analysis

We utilized a seizure detection protocol that had been previously described by our lab (Bar-Klein et al., 2014). Briefly, using an in-house MatLab software program, electrocorticographic signals were processed to detect epilepsy using a combination of five feature extraction (energy, curve length, standard deviation, relative power in the beta and low gamma frequency bands) to set an artificial neural network (ANN). A 1.5 long ECoG segment from a pilocarpine, genetic and albumin models of epilepsy (Milikovsky et al., 2017) were used in training the features to detect epilepsy at a set ANN. To reduce the false positive rate of seizure detection, ANN was set at 1 such that events persistently above the set threshold with duration lasting at least 5 seconds were classified as seizures and also events within the next 60 second window were considered part of the initial event. Data

were buffered into 2 seconds epochs with 1 second overlap having a bandpass filtered at 2 – 100Hz. Similarly, a periodogram Fourier transform using an in-house MatLab script was used to calculate the average power spectrum for the length of each recording for each animal and this was later normalized for each power bandwidth using Microsoft Excel (Tomkins et al., 2011). Finally, for slow wave events, the median power frequency (MPF) was extracted from each epoch using an in-house MatLab script and this metric was used in determining the number and duration of slow wave events per animal.

2.9 Perfusion

After all testing is complete, animals were euthanized and perfused. The protocol involved an intraperitoneal injection of sodium pentobarbital. We checked the pedal reflex at intervals of 1-2 minutes till the animal is no longer responsive. A longitudinal incision is made from the xiphoid process through the right and left rib cage to expose the heart. A cannula connected to the perfusion pump was then placed into the left ventricle and clamped. The tubing on the other end of the perfusion pump is inserted in normal saline and the pump switched. Micro scissors are then used to snip the right atrium and the animal is perfused for 4 minutes or till perfusion is adequate. The tubing is then placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and tissues fixed for 4 minutes. The scalp and skull were cut open and brain extracted. Photographs of the brain were then taken (Figure 6).

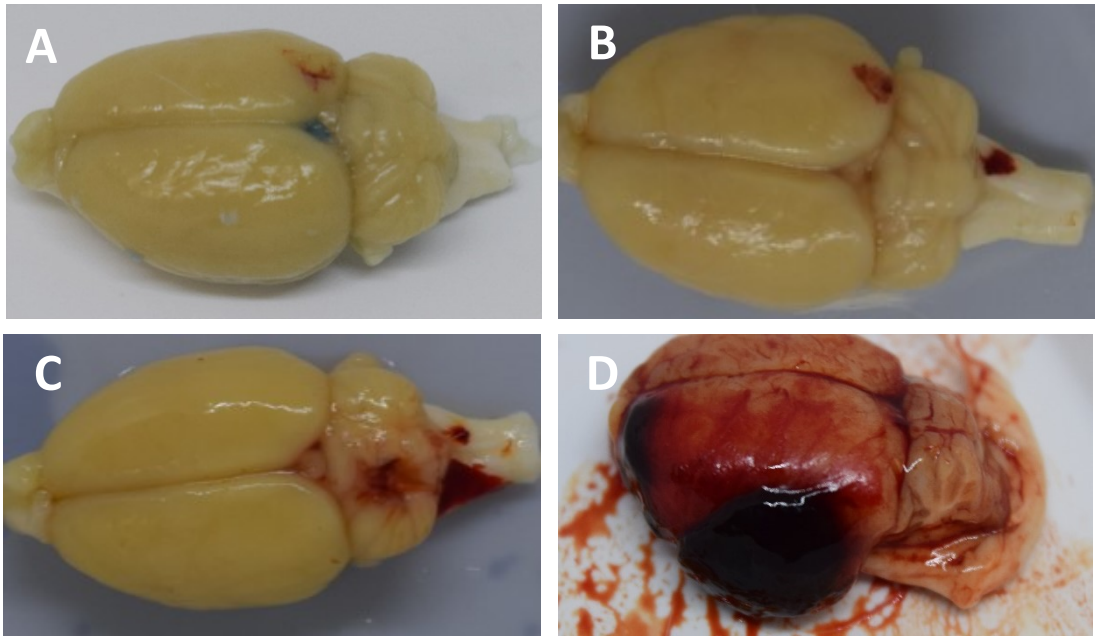

Figure 6. Structural brain changes after mTBI (A) Contusion of the right posterior parietal cortex. (B) Contusion on the right posterior parietal cortex and subarachnoid hemorrhage. (C) Contusion on the cerebellum, spinal cord and Subarachnoid hemorrhage. (D) Subdural hematoma of the left frontal and left temporal cortex.

2.10 Statistical Analysis

All data were analyzed using Ethovision, MatLab and Graphpad Prism software. The Mann-Whitney non-parametric test was utilized to test for statistical significance for BBBD between TBI and control animals, power spectrum dynamics between TBI and controls, paroxysmal slow wave events between TBI and controls, and behavioural scores in TBI and control animals while Holme-Sidak multiple t test was used for evaluating weight changes between TBI and control animals. Pearson's correlation test was also utilized to examine the relationship of BBBD with cognitive impairment and average frequency of slow wave events per day. Fisher's exact test was used to examine the relationship between acute convulsive seizures and mortality on impact. A P value was set at < 0.05 for statistical significance and all data were presented as mean ± SEM.

CHAPTER 3: RESULTS

3.1 Establishing the Weight Drop Model of Moderate Traumatic Brain Injury

3.1.1 Mortality on Impact

A total of 39 rats (controls, 9; TBI, 30) were used to establish the weight drop moderate TBI model. Of the 30 TBI rats, 15 were hit with a weight of 450g at a height of 1.2 m and the remaining 15 with a weight of 500g at the same height. In the group of animals hit with 450g, 3 died on impact representing a mortality rate of 20% while 4 animals died on impact in the group of 500g representing a mortality of 26%.

3.1.2 Neurological Severity Scoring

Neurological severity scores were calculated and reported in Figure 7 for each animal at baseline, 20 minutes, 24 hours, 48 hours and 1week post TBI. A Mann-Whitney test was performed on the composite scores for each group, at each time point. There was no significant difference in neurological scores between all three groups at baseline (control, n = 9; TBI 450g, n = 15; TBI 500g, n = 15). TBI animals in the 450g weight group had significantly lower scores compared to controls at 20 minutes post TBI ($P = 0.0063$). However, animals hit with 500g had a lower neurological score compared to controls, but this was not found to be statistically significant. The animals in both weight groups recovered at 24, 48 hours and 1 week and no statistically significant difference was observed between the TBI groups and controls at these time points.

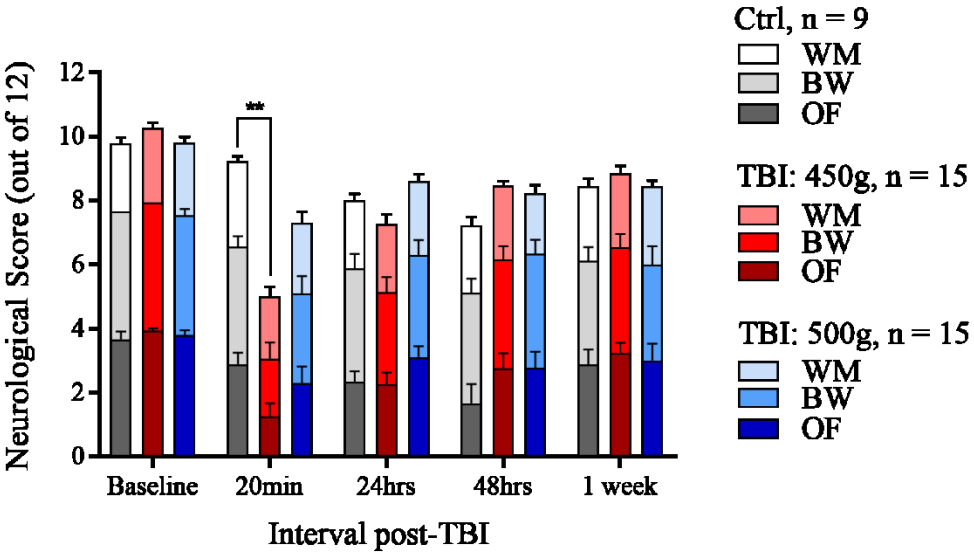

Figure 7. Average neurological scores at varying time points. Neurological score at 20 minutes was significantly reduced for the 450g weight group compared to control ($p = 0.0063$). The same effect was not observed at 20 min for the 500g weight group. No statistical difference was observed for the three groups at 24, 48 hrs and 1 week.

3.2 Assessment of Acute Injury (0-7 days)

3.2.1 Early Consequences of mTBI

Based on the experimental results observed during validation of the mTBI model as previously described, we used a weight of 450g and height of 1.2m based on the observed mortality and neurological deficits we observed for the induction of injury in order to investigate the effect of moderate TBI on acute morbidity and mortality.

3.2.1.1 Mortality and Acute Convulsions

Figure 8a and 8b show the percentage of mTBI animals that died and had acute convulsions immediately post-injury induction. For mortality on impact, 17 of the 65 TBI rats died instantly, representing 26% mortality. Of the 48 rats who survived TBI, 23 (48%) had acute convulsions during the 20-minute recuperation time allotted to each animal.

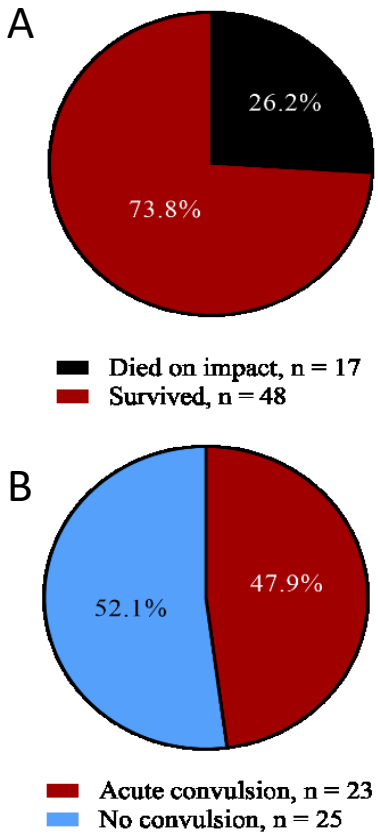

Figure 8. Mortality and acute convulsion post TBI. (A) Mortality rate of 27% was observed post TBI. (B) 48% of the animals had acute convulsions (aC+) while 52% had no convulsions (aC-).

3.2.1.2 Neurological Deficits and Weight Loss

Injury severity was also assessed by tracking the animals neurological behavioural scores and weight over time (Figure 9a). Consistent with the neurological score findings during the model validation experiments as previously described, neurological scores were significantly worse at 20 minutes among the TBI animals compared to control ($p = 0.0067$, Mann-Whitney test). However, TBI animal performance on the administered neurological tasks began to improve within 24 hrs post-TBI as no differences were observed between

TBI and controls at this point ($P < 0.33$, Mann-Whitney test). Further neurological testing performed at 48 hours and 1 week post-impact also found no differences between control and TBI animals (all $P > 0.05$, Mann-Whitney test).

Animal weights were also monitored during the course of the experiment. Weight is a good measure that can be used to assess failure to thrive. As part of our experimental protocol, animals who lost more than 10% of their body weight in less than 24 hrs were removed from the study. mTBI animals show a significant weight change at 24 hrs post-TBI compared to control ($P < 0.0001$, Holm-Sidak multiple t test), representing about 3% weight loss. However, TBI animals appear to recover at 1-week post injury but were still observed to lag behind healthy controls ($p < 0.0001$). On further testing, no significant difference in weight was observed between TBI and control animals at 1-month (Figure 9b and 9c).

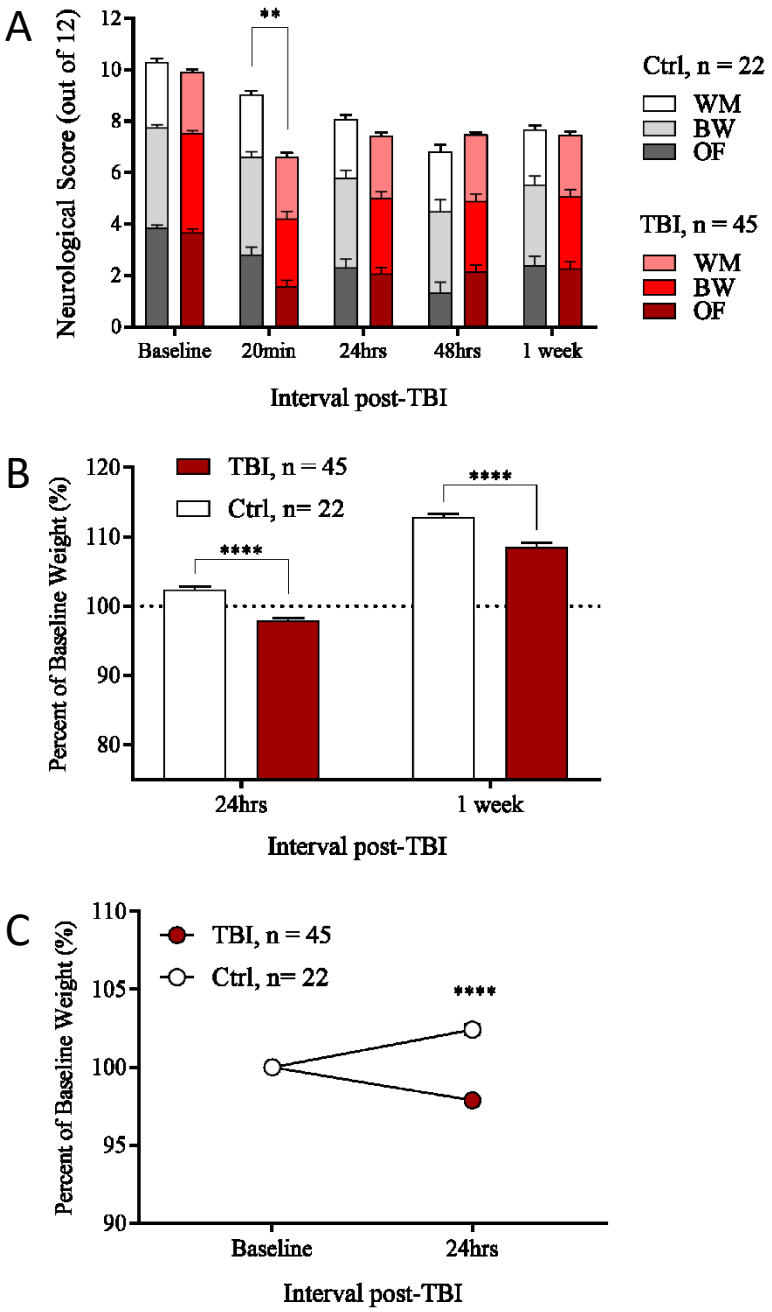

Figure 9. Average neurological scores and weight changes at varying time points. (A) Neurological score at 20 minutes was significantly reduced for TBI animals compared to control ($p = 0.0067$). No significant difference was observed between control and TBI at 24, 48 hrs and 1 week ($P > 0.05$). (B) TBI animals show a significant weight change at 24 hours ($p < 0.00001$) and 1 week ($p < 0.00001$) but recover at 1 month. (C) The weight change observed in TBI rats represents a 3% weight loss.

3.2.2 Animals with Acute Convulsion showed increased Post-Impact Mortality, Poor Neurological Scores and Weight Loss

We next examined the relationship between animals with an acute convulsion and the risk of mortality following TBI. As previously shown, a total of 17 rats died on impact out of 65 TBI animals which represents a 27% mortality rate. Of the 17 animals that died on impact, 14 (82%) had post-impact convulsions while 23 (47%) of the 48 animals who survived the TBI had acute convulsions. Hence, 62% of animals with convulsion survived while 89% of animals without convulsion survived and this was found to be significant (Figure 10a) ($p < 0.05$, Fisher Exact test).

Further testing also revealed that acute convulsion results in poor performance in the administered neurological task. A significant number of animals with an acute convulsion (aC+) post-TBI had lower composite neurological score at 20 minutes compared to the no convulsion (aC-) ($p = 0.04$, Mann-Whitney test) and control groups ($p = 0.003$, Mann-Whitney test) (Figure 10b). However, a similar trend was not observed for the weight change between animals with convulsion, no convulsions and control (Figure 10c).

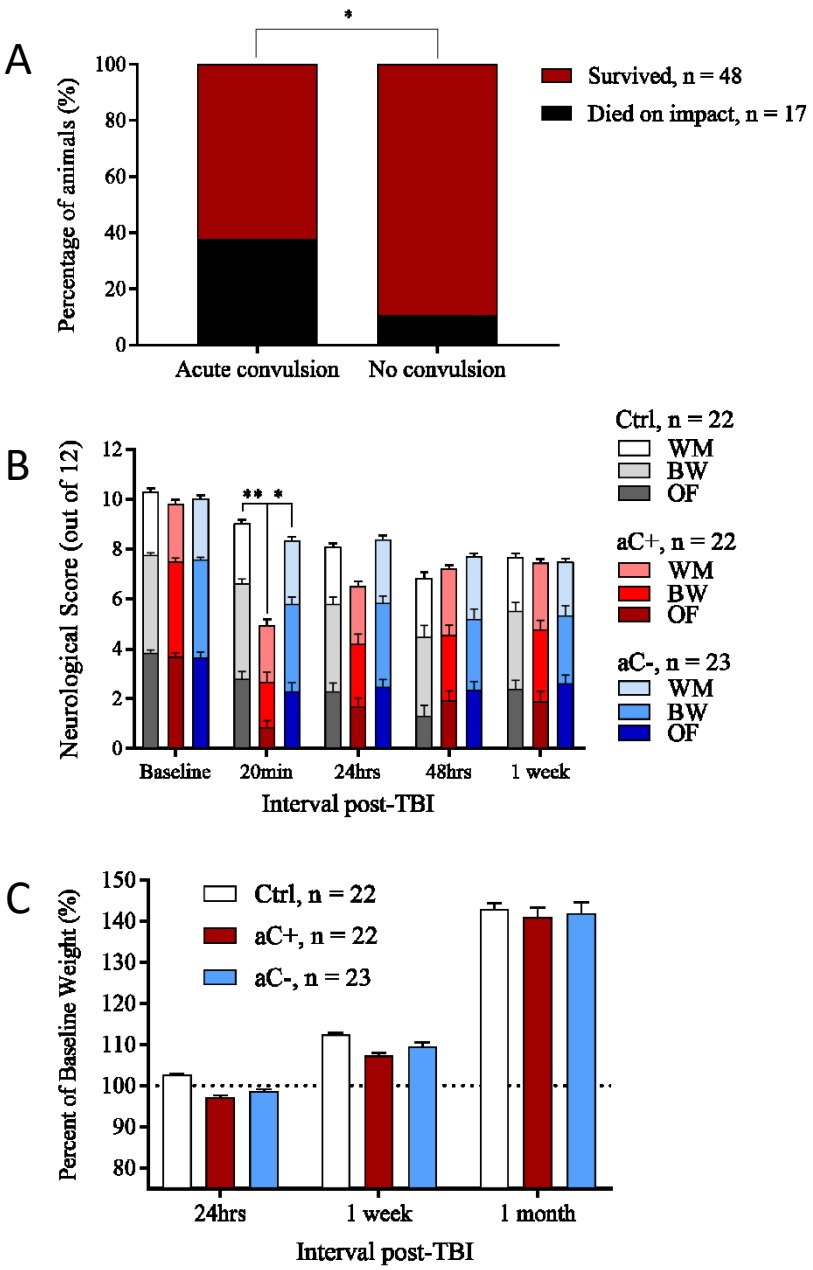

Figure 10. Acute convulsion, neurological score and weight change. (A) Acute convulsion and post-impact mortality. A significant number of animals with convulsion died on impact compared to those without convulsion ($p < 0.05$, Fisher's exact test). (B) A statistically significant number of animals with a convulsion had low neurological scores at 20 minutes compared to the control ($p = 0.003$) and no convulsion group ($p = 0.04$, Mann-Whitney test) (C) No difference was observed between animals with convulsion, no convulsion and control groups at 24 hours, 1 week and at 1 month for percent of baseline weight.

3.3 Blood-Brain Barrier Imaging

3.3.1 TBI Animals Show BBB Disruption at 48 hr post-TBI

We conducted MRI at 48 hrs (TBI, 22; Ctrl, 5) and 1 month (TBI, 30; Ctrl, 5) time point to detect and quantify blood-brain barrier disruption (BBBD) which occur following TBI. The BBB permeability detected on MRI were quantified as a measure of the detectable lesion observed in control animals, and same slope values were then applied to TBI animals. We observed that a slope value of 0.00043 showed a considerable separation between both control and TBI animals using a K mean clustering and this value was chosen as the threshold for pathological voxels. A cumulative frequency graph of all the calculated slope values for control and TBI animals at 48 hr and 1 month revealed increased BBBD in TBI rats at 48 hrs, a similar rightward shift was not observed for the TBI animals at 1 month (Figure 11a). To test if a significant number of animals demonstrate this microvascular opening at 48 hrs, we performed a Mann-Whitney test between these groups. Indeed, a statistically significant increase in BBBD was observed in TBI animals at 48 hrs ($P = 0.02$, Mann-Whitney test).

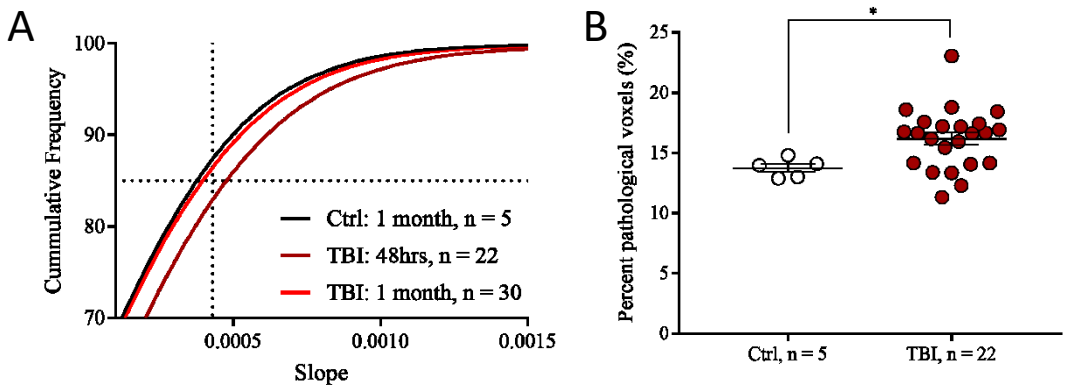

Figure 11. Cumulative frequency curve of all slope values and percent of voxels with a slope above 0.00043. (A) TBI animals at 48 hr show BBB disruption but not at 1-month (B) Percent pathological voxel was significantly higher for TBI animals at 48hr (P = 0.02, Mann-Whitney test).

3.3.2 Blood-Brain Barrier Integrity Recovers 1-Month Post-Injury

We further explored the effect of time on the BBB by repeating the MRI at 1 month of percent pathological voxels at 48 hrs and 1 month for the respective TBI and control animals. Interestingly, we found a significant reduction in the percent of pathological voxels in TBI animals ($P < 0.0001$, Wilcoxon match-pair signed rank test) (Figure 12). However, a significant difference was not seen in control animals (Figure 12).

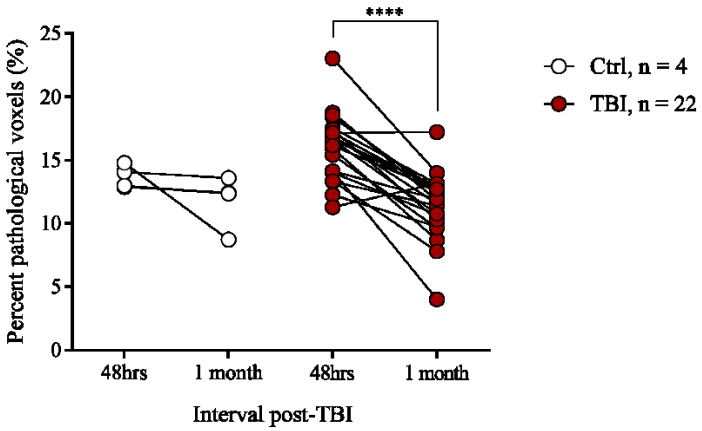

Figure 12. TBI Animals show recovery from blood-brain barrier dysfunction at 1-month post-injury.

3.4 Delayed Complications

3.4.1 TBI Animals Are Cognitively Impaired

Novel object recognition and Morris water maze tests were used to assess serial learning and spatial memory. For the novel object recognition test, latency to novel object and distance moved before interacting with the novel object were the two parameters used to assess cognition. Control and TBI animals displayed no preference in interacting with the novel object in both analyzed tasks and as a result, no difference was observed between groups ($p > 0.05$, Mann Whitney test) (Figure 13a and 13b). Animals in control and TBI group also failed to show exploratory interest for familiar objects during the sample phase of the test. We then employed the more sensitive forced swimming test- the Morris water maze to evaluate the animals for cognitive deficits. Cumulative duration and mean distance to the platform were analyzed using the Ethovision software. TBI animals travelled a significantly longer distance compared to control for the immediate probe ($p = 0.02$, Mann-Whitney test), however, there was no significant differences observed when the probe trial was administered 24 hours later (Figure 13c) ($p > 0.05$, Mann-Whitney test). The

cumulative duration spent interacting with the novel object was significantly shorter in TBI animals compared to control for the immediate probe (p = 0.04, Mann-Whitney test). The same trend in cumulative duration was not observed at the 24 hr time point (Figure 13d)

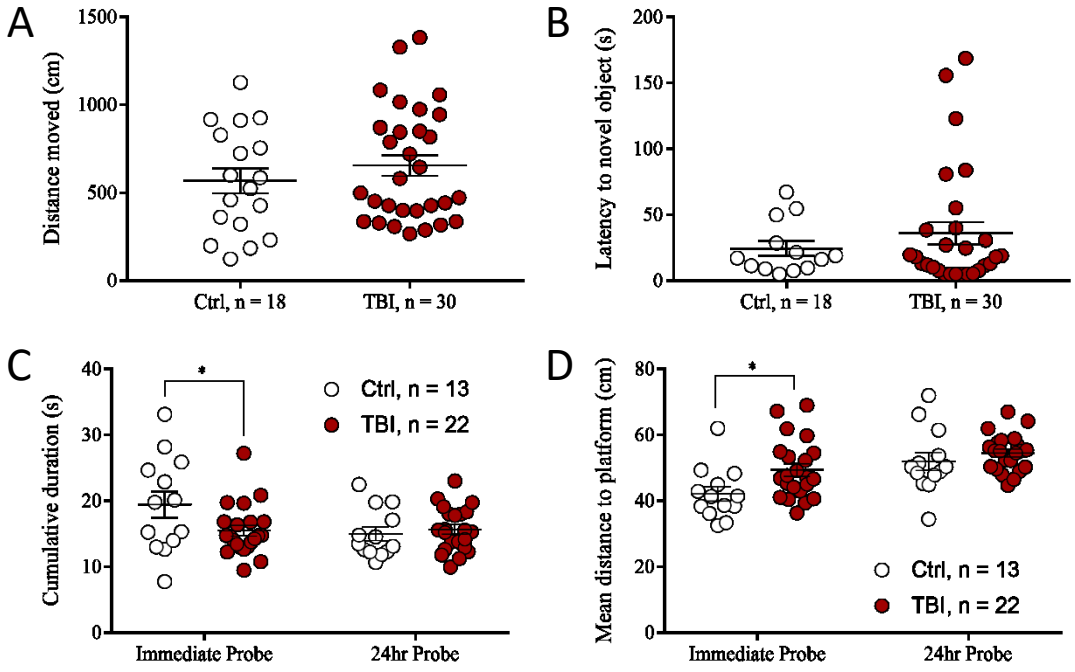

Figure 13. Cognitive tests: novel object recognition and Morris water maze tests. (A) Total distance moved (B) Latency to novel object (C) Cumulative duration in the platform arena for the immediate and 24 hr probe trial (D) Mean distance to platform arena for the immediate & 24 hr probe trial.

3.4.2 Electrocorticographic Events Following mTBI

3.4.2.1 Post-Traumatic Epilepsy (PTE)

Continuous telemetric video ECoG recordings were monitored in 35 animals (TBI, 23; Control, 12) starting from 8 weeks post-TBI. 6-months post-TBI, 6 out of 23 (26%) TBI rats developed PTE (Figure 14a).We found a progressive increase in the number of animals who developed PTE: 2 (8%) out of 23 TBI animals during the first recording cycle

developed PTE and an additional 4 (17%) TBI animals developed PTE in the second cycle (Figure 15a). The average frequency of seizure occurrence also evolved from an average daily seizure of 12 per day at day 63, to 18 per day at day 104 post-injury induction (Figure 15b). The mean number of seizure events per day was 11.9 throughout the recording. Additionally, seizure duration remained between 7 to 20 seconds and the seizure phenotype was most often a behavioural arrest or freezing behavior. Most of these seizures were observed to be more prevalent during the dark phase (74%) than the light phase (26%) of the facility light-dark cycle. Spontaneous seizures (electrocorticographic or behavioural) were not found in any of the control rats.

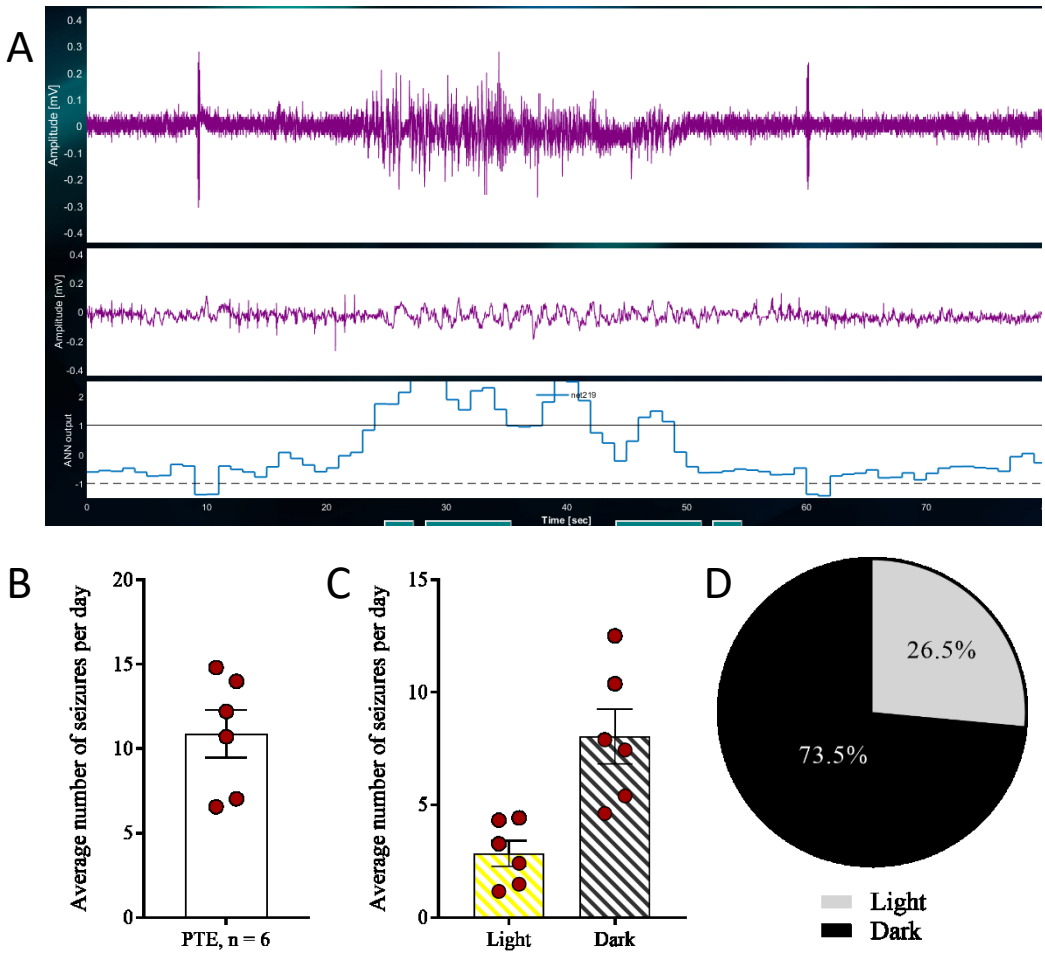

Figure 14. Example of a seizure and seizure frequency per day (A) Example of a seizure detected by the in-house seizure-detection software (B) The mean number of seizures per day (C) The average number of seizures during the 12hr light-dark cycle (D) Diurnal variation of seizures in the epileptic rats.

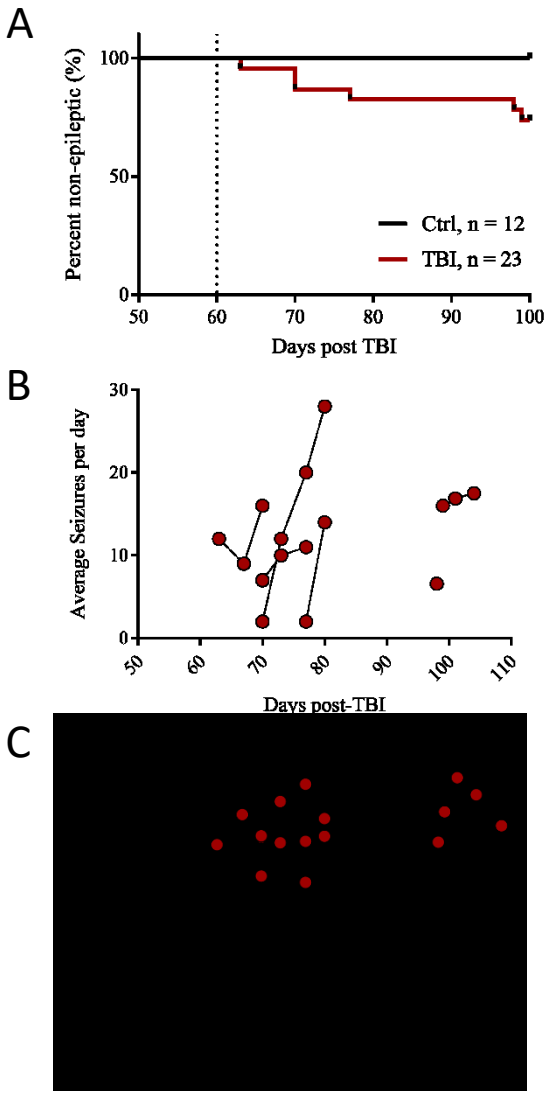

Figure 15. Characterization of seizure after mTBI (A) Percentage of TBI animals with PTE at different experimental time points (B) Average seizures per day for each of the 6 PTE rats (C) Duration of seizures amongst the 6 PTE rats over time.

3.4.2.2 Spectral Analysis Revealed Slowing of Background ECoG activity Epileptic Animals

To test whether epileptic discharges in the brain of TBI animals are associated with background slowing of ECoG activity, we compared the fast Fourier transform (FFT) power spectrum analysis among epileptic, non-epileptic and control animals. A significant

increase in theta power bandwidth was observed between epileptic TBI and control animals ($p < 0.0001$, multiple t test), epileptic and non-epileptic TBI animals ($p<0.0001$, multiple t test), as well as between non-epileptic TBI and control animals ($p< 0.01$, multiple t test). No difference was observed for the other power bandwidths (Figure 16).

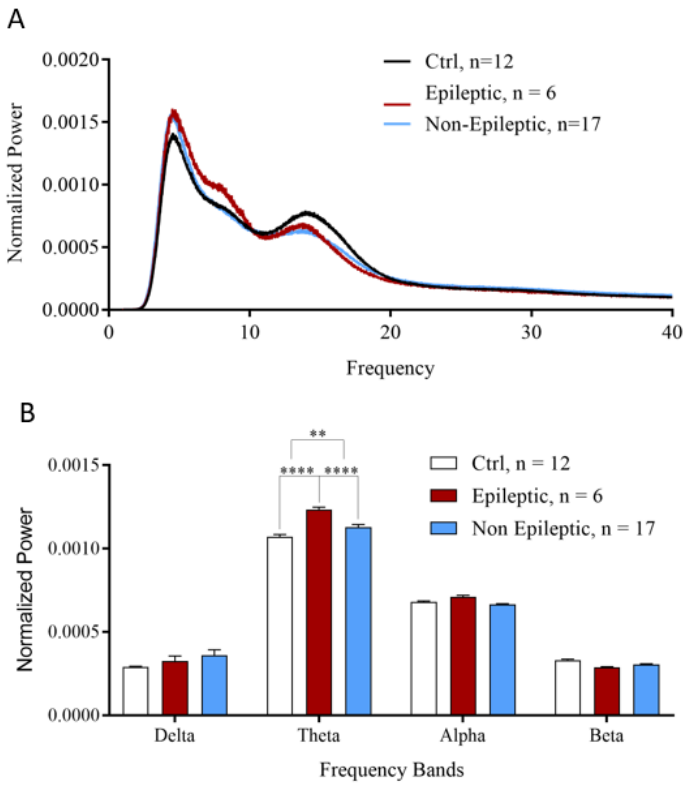

Figure 16. Abnormal slowing of background ECoG activity (A) Average normalized power of control, epileptic and non-epileptic animals (B) Averages of normalized power spectrum bandwidths showing a significant increase of theta bandwidth in epileptic animals compared to non-epileptic and control animals ($p < 0.0001$, multiple t tests) and between non-epileptic and control animals ($p < 0.01$, multiple t test).

3.4.2.3 TBI Animals Display Increased Frequency of Slow Wave Events

In search for a non-invasive ECoG biomarker which can accurately reflect epileptogenic animals, we tested the occurrence of an emerging concept termed a paroxysmal slow wave event (PSWE) (see introduction). Increased PSWE had been previously described in humans with mild cognitive impairment and Alzheimer's disease as well as in ageing mice (Milikovsky et al., accepted). A sample trace of a PSWE detected in a patient with Alzheimer's disease is shown in Figure 17. Analysis of the average PSWE per day between TBI and control animals showed a significant increase in frequency of these events in TBI animals ($p < 0.05$, Mann-Whitney test). The number of events appear to show a declining trend over time in the TBI group, however, no significant difference was observed between TBI and control for the average PSWE over time. We next tested if PSWEs could reflect an underlying neuronal hypersynchrony. Indeed, the average number of slow wave events in 5 of the 6 (83%) epileptic rats ranged from 10 to 40 per day, as opposed to < 6 in all control and most of the non-epileptic rats. Kruskal-Wallis test with Dunn's multiple comparisons showed a significant effect ($p = 0.01$) and a significant difference between control and epileptic animals ($p = 0.008$). Two of the remaining 18 non-epileptic rats had 10 or more slow events per day and are being monitored to a later time point to determine if they will develop epilepsy (Figure 18).

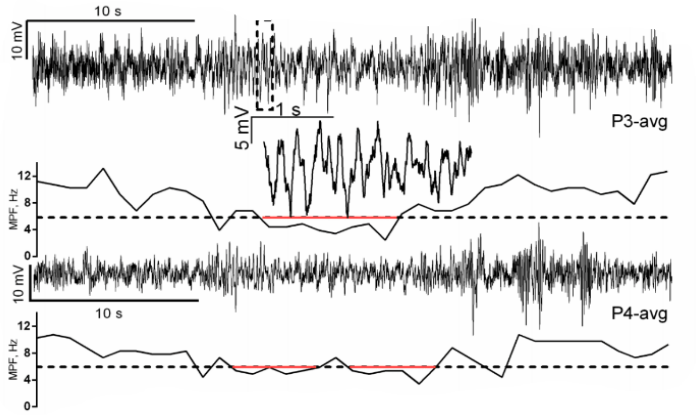

Figure 17. A sample trace illustrating PSWE. The trace shows PSWE detected in an Alzheimer's disease patient (Milikovsky et al., accepted) from electrodes P3-avg and P4-avg (reference trace). Below each trace is the Median Power Frequency (MPF). PSWE is represented as segment of the trace below 5 Hz (red).

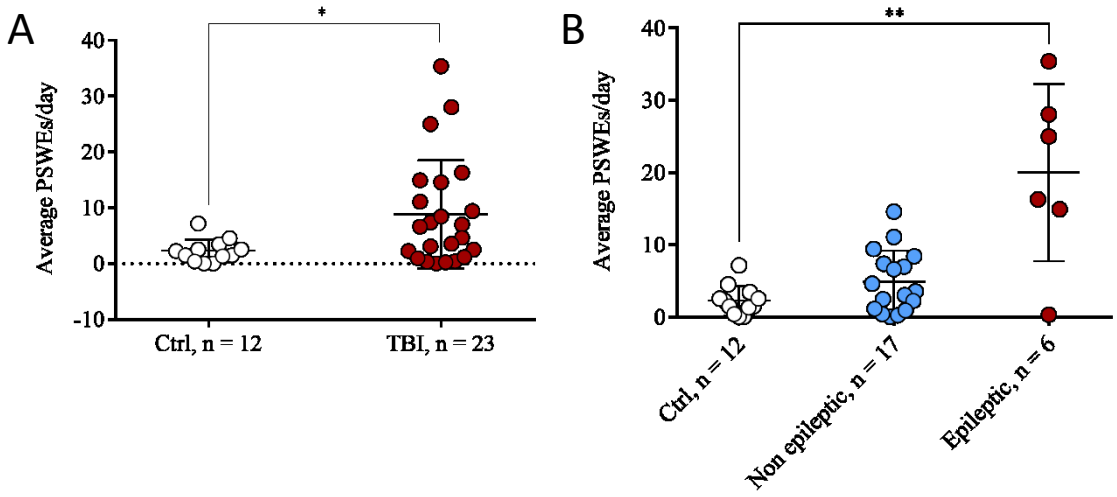

Figure 18. PSWE per day in control and TBI animals (consisting of epileptic and non-epileptic groups (A) Mean PSWE per day in control and TBI animals. The TBI rats show significantly increased number of daily slow wave events ($p < 0.05$) (B) Number of PSWE in rat subgroups; epileptic, non-epileptic and control. Note that 5 out of the 6 epileptic rats have more than 10 daily events and this was found to be significant compared to healthy controls ($p < 0.01$).

3.5 Does Blood-Brain Barrier Predict PTE and Its Co-Morbidities?

An overwhelming body of evidence has previously demonstrated that BBB dysfunction promotes epileptogenesis and ultimately seizure initiation (Bar-Klein et al., 2014; Marchi et al., 2007;Tomkins et al., 2011), as well as cognitive impairment (Wang & Li, 2016). Since the BBB operates in the realm of the brain neuronal circuitry, an extrinsic perturbation of the neuronal-vascular interface that occurs following mTBI could predict the development of epilepsy and its co-morbidities. The current study favours the plausibility that BBB disruption mirrors the evolution of PTE and cognitive impairment and thus may serve as a potential non-invasive biomarker. To answer one of the hypotheses raised in this study, we examined the relationship between the magnitude of BBB disruption in control, non-epileptic and epileptic animals (Figure 19a). Although we found a significantly increased BBBD as reflected in the percent pathological voxels in TBI animals compared to control, a similar difference was not observed on further stratification of these epileptic animals into epileptic and non-epileptic subgroups (Figure 19a). We further explored the relationship of BBBD and PSWE. This possibility was worth evaluating based on our findings (Figure 15c) that PSWE might reflect an underlying neuronal hypersynchrony. The result revealed that the volume of BBBD is not correlated with the average number of PSWE events per day (Pearson correlation test, $r^2 = 0.1014$; $p = 0.17$) (Figure 19b). We examined the relationship between the magnitude of BBBD in TBI and control animals and severity of cognitive deficits observed in the Morris water maze test during the immediate probe trial. A positive correlation was demonstrated between the severity of short-term memory deficits as measured by the immediate probe

trial and the magnitude of BBB disruption as measured by the percent pathological voxels ($r^2 = 0.2261$; $p = 0.02$) (Figure 19c).

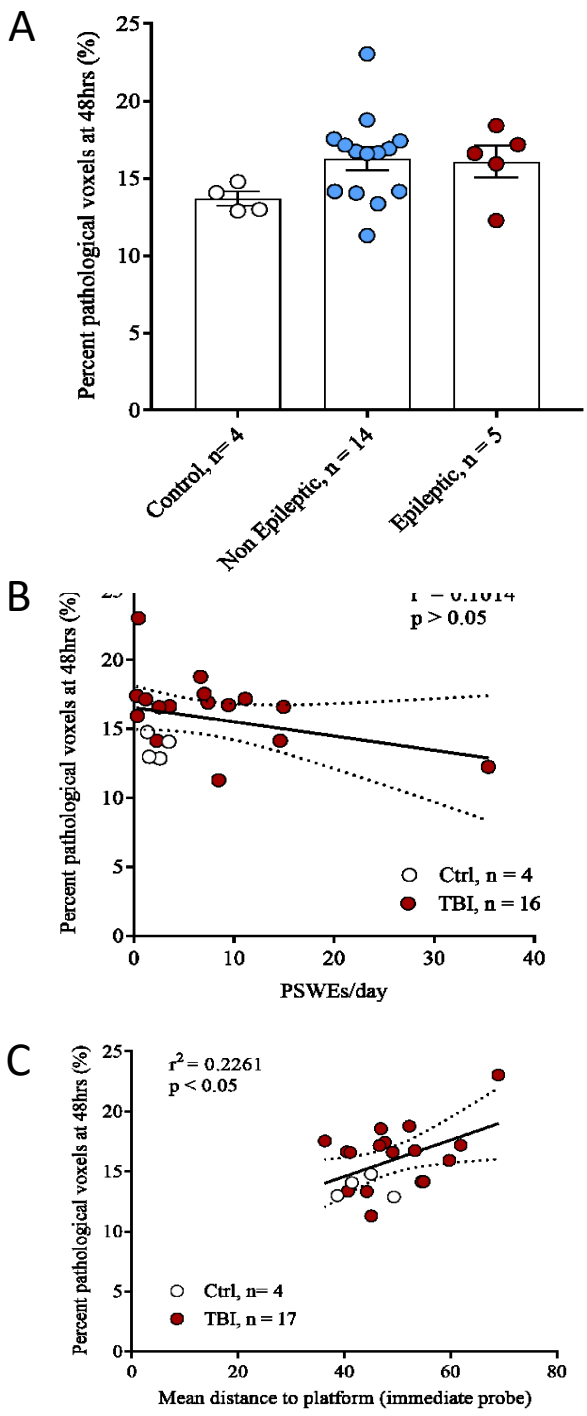

Figure 19. Blood-Brain Barrier Correlations (A) Relationship between magnitude of blood-brain barrier disruption and PTE (B) Relationship between magnitude of blood-brain barrier and average number of paroxysmal slow wave events per day (C) Relationship between magnitude of blood-brain barrier disruption and learning deficits.

CHAPTER 4: DISCUSSION

TBI and the long-term complications of PTE and cognitive impairment are becoming a global epidemic. Complications can develop months to years following the primary insult to the brain. The search for a biomarker is currently the centerpiece of PTE research and the role of neuroimaging to detect BBBD following TBI has revealed great insights as a potential non-invasive biomarker. As defined, Pitkanen et al. (2019) aptly describes a biomarker as a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions"(Pitkänen et al., 2019). There is currently no predictive biomarker for early detection of epileptogenesis following TBI (Pitkänen et al., 2019). The "standard of care" is mainly targeted toward acute symptomatic management and the currently available drugs are ineffective in preventing PTE (Wilson et al., 2018). When PTE ensues, it is often pharmacoresistant (Klein et al., 2018; Webster et al., 2017) and associated with significant morbidity and neuropsychiatric complications (Wijayatilakea et al., 2015). Hence, a predictive biomarker would hold much promise to facilitate the identification of TBI survivors at high risk of developing PTE and its co-morbidities.

In this thesis, I developed a weight drop model of mTBI which recapitulates some of the mechanism(s) of acquisition of human TBI. The TBI animals can be closely monitored and assessed during the acute and delayed time points for the development of complications (McAteer et al., 2016), as would be done in a human patient who experienced a mTBI. Not only were the animals followed over time, but the animals also had a contrast-enhanced MRI to detect and quantify the extent of the disruption of the BBB. We worked with the hypothesis that the magnitude of BBB disruption may be a predictor

for the development of PTE and its co-morbidities. Our working hypothesis was hinged upon previous experimental findings from our lab (Friedman, 2011; Milikovsky et al., 2017; Serlin et al., 2019) and others (Dadas & Janigro, 2019; Zenaro et al., 2017) where BBB dysfunction was found to be a common phenomenon shared by different brain diseases including TBI (Weissberg et al., 2014).

My main findings are that (1) transient significant leakage of the BBB occurs at 48 hours post-TBI with BBB integrity recovering at 1-month; (2) positive significant correlation between BBB dysfunction and impairments in learning and short term memory; (3) this model produces PTE at rate of 26%, with mostly a focal phenotype; (4) increased network ECoG slowing in epileptic animals compared to non-epileptic injured or control animals (5) increased occurrence of paroxysmal slow wave events in animals with PTE. The experimental findings in support of these conclusions are discussed below.

4.1 The Model

In developing the mTBI model, we adapted the Mamarou impact acceleration weight-drop model in order recapitulate the acceleration-deceleration and rotational head movements characterizing the mechanisms of injury acquisition in humans (Büchele et al., 2015; Siebold et al., 2018). Consequently, we showed that a weight of 450g falling from a height of 1.2m modelled the neuro-behavioural symptomatology expected for moderate TBI (Hou et al., 2017). We employed three reported criteria shown to reflect moderate head injury severity in rodents: (1) We observed an injury-induced immediate post-impact mortality of 20%, which was consistent with mortality rates from previous studies of 21-30% (Andriessen et al., 2011; Frey, 2003; Manley, 2013). (2) The presence of pronounced

neurological deficits at 20 minutes post-injury induction as reflected by performance on the administered composite neurological tasks. We found that TBI animals had substantial deficits in combined neurological tasks compared to control animals, a finding consistent with what was observed for mTBI in a rat model (Manley, 2013; Tagge et al., 2018). However, TBI animals recovered to their premorbid states within a week. Contrary to my experimental observation, Hou et al (2017) showed a progressive deterioration in the administered neurological task at 1 week (Hou et al., 2017). (3) In addition to the above criteria, gross pathological brain changes like contusions, subarachnoid hemorrhage, and subdural hematoma were observed in the brain of a subset of animals that were sacrificed due to their inability to perform the neurological task. These anatomic brain changes are in keeping with other studies using mTBI (Maas et al., 2008). We next used our established model to answer our hypothesis- the role of blood-brain barrier pathology in PTE. Moderate TBI has been shown to produce long-lasting and assessable acute and chronic disabilities.

4.2 Neurobehavioural Outcomes of Primary Injury

We proceeded to test neuro-behavioural outcome of the acute injury in 65 injured rats. An interesting observation was the presence of acute post-impact convulsion in 48% of the rats. Acute immediate post-impact convulsion manifested as repetitive hind-limb kicking, tail movement and muscle spasm. This observation was consistent with Marmarou's 1994 experimental findings in a rat weight drop model of TBI (Marmarou, 1994). Also, animals with immediate convulsions were more likely to die compared to those without convulsion. A previous study revealed that an explanation for the observed

mortality could be depression of the central respiratory drive following an acute convulsion (Marmarou, 1994). Marmarou (1994) further showed a decreased mortality rate in mechanically ventilated animals during impact.

Also, consistent with the results from our weight validation experiment, we notably showed a deterioration in neurological function at 20 minutes in the 48 TBI animals compared to control animals, an observation that could reliable reflect an underlying primary injury of a moderate severity. A similar decline in acute neurological outcome was previously demonstrated in humans following moderate to severe traumatic brain injury (Rosenbaum et al., 2018). However, a significant effect of time was observed in which TBI animals began to recover from the primary brain injury starting at 24 hrs, 48 hrs and full recovery at 1-week post TBI. It is noteworthy to mention that all animals were given 3 mLs of subcutaneous ringer lactate and mash after the 20-minute neuromotor behavioural assessment to aid in recuperation. Without nursing these animals, there is a higher likelihood that more animals could become severely morbid and may suffer sudden death, an observation we found in two TBI animals. Our nursing paradigm closely parallels a clinical setting in which moderately head injured patients go through a period of active rehabilitation post-injury (Rosenbaum et al., 2018). Given our previous observation in which we showed a higher mortality rate in TBI animals who experienced a post-impact convulsion, we next sought to determine if the occurrence of a convulsion could predict neurological outcome as objectively assessed by the neuromotor behavioural tasks earlier described. Indeed, post-impact convulsions were associated with lower neurological outcome at 20 minutes compared to a subgroup of TBI animals without convulsion and control. To the best of my knowledge, this is the first study showing a relationship between

immediate post-impact convulsion and acute neurological outcome in a moderate weight drop model of TBI. These findings suggest that the occurrence of acute convulsions could reflect morbidity post-injury. Future studies should investigate the relationship between both phenomena.

We observed that 24 hrs post-injury, TBI animals lost more weight than healthy controls. This weight change was not long-lived as rapid recovery in weight was observed at 1-week and these animals continued to thrive at 1 month and through the remainder of the experiment. As previously described, it is important to note that the recovery process may have been aided by our fluid and mash intervention to all the animals. While moderate TBI could be ultimately fatal without interventions, our results suggests that early nursing could lead to faster recuperation similar to what has been reported in human subjects where early rehabilitative care resulted in a notable functional recovery (Rosenbaum et al., 2018). In line with acute weight change observed in TBI animals, we next examined if the occurrence of an immediate post-impact convulsion had an effect on the observed acute weight loss. Contrary to the observed finding for the neurological score, our result does not show an effect of convulsion on weight change. A possible explanation for this observation could be that different brain centers mediate appetite and neurological performance such that both events are non-mutually exclusive.

As discussed above, a subset of animals with poor neurological scores at 24 and 48 hrs and also a significant amount of weight loss despite interventions, and were sacrificed and perfused 48 hrs after mTBI induction. We observed heterogenous gross pathologies in the brain: subarachnoid hemorrhage, subdural hematoma, and contusion. Human studies have also revealed a heterogeneity in gross pathologic lesions observed following moderate

to severe TBI (Badaut et al., 2019; Sarkar et al., 2014). A commonality shared amongst human and rodents could be that the presence of blood volumes prognosticates severity of injury (Badaut et al., 2019).

4.3 Blood-Brain Barrier Dysfunction

The BBB has been shown by several studies in rodents and humans to be dysfunctional following traumatic brain injury (Tagge et al., 2018;Tomkins et al., 2011). Previous studies have reported that opening of this vascular-neuronal interface occurs within hours post-impact (Shlosberg et al., 2010), peaks at 48 hrs (Hakon et al., 2015) , and recovers days to weeks following the injury (Shlosberg et al., 2010). Consequently, a perturbation in the BBB has been shown by several studies to result in PTE and cognitive deficits (Dadas & Janigro, 2019; Reid et al., 2016;Tomkins et al., 2008; Zenaro et al., 2017; Zlokovic, 2008). Hence, we sought to address a pertinent question in the search for a biomarker- could BBBD predict the development of PTE and its co-morbidities?

We showed a pronounced increase in BBBD in TBI rats compared to controls. These experimental findings were congruent with previous studies carried out in rats and human subjects (Ivens et al., 2007;Tomkins et al., 2011; Weissberg et al., 2014). MRI 1-month post-injury did not show persistent opening of the BBB but showed repair at this time point. However, 13% of the TBI animals with a 1 -month MRI showed persistent BBB permeability as reflected in their percent pathological voxels above the slope. This result is in accordance with reports from previous studies where a long-lasting BBB opening was observed in a subset of experimental cohorts for both moderate and severe TBI (Tomkins et al., 2011).

4.4 Delayed Brain Dysfunction as Revealed by Cognitive Tests

As stated previously, TBI is a progressive disease and a recognized risk factor for the development of neurodegenerative diseases like Alzheimer's disease in humans and rodents (Tajiri et al., 2013; Wang & Li, 2016). Premised on this, we next evaluated the evolution of long-term complications in a subgroup of TBI rats using two cognitive tests: Morris water maze and novel object recognition. Although, multiple cognitive tests exist, previous studies on TBI and cognitive impairment in humans incorporated tests that can appropriately detect the symptomatology of cognitive impairment like memory loss as well as attention deficits (Millis et al., 2001). In this line, the Morris water maze and object recognition tests have been shown by previous studies to be sensitive in evaluating cognitive deficits in rodents (Karl et al., 2012; Tucker et al., 2018). Furthermore, the likelihood of developing cognitive impairment in moderate-severe TBI more than doubles that of mild injury (Vincent et al., 2014; Wang & Li, 2016).

Indeed, our findings suggest that TBI rats showed significant impairment in short term spatial memory when compared to healthy controls. This was reflected in the immediate probe trial compared to a healthy control for the mean distance to platform and cumulative duration in the platform arena. Conversely, our findings also suggest that TBI had no effect on long term memory as seen in the 24 hr probe trials. This result is consistent with the Cho et al., 2013 study, which also found short-term cognitive deficits in a blast model of moderate TBI in rats (Cho et al., 2013). A plausible explanation for this finding could be that these animals are developing an Alzheimer's like disease where short-term memory is lost first and then followed in the long run by impairment of long-term memory-

and this correlates closely with a hippocampus-specific deficit which is well known to be susceptible to TBI (Hou et al., 2017). Several publications have given credence to the aforementioned assertion that moderate to severe TBI can lead to the development of Alzheimer's disease and other neurodegenerative diseases (Julien et al., 2017; Kokiko-Cochran & Godbout, 2018; Ramos-Cejudo et al., 2018). Contrary to our observation with the MWM, we found no difference between TBI and control animals for NORT. This could stem from methodological differences in our NORT experimental design where we carried out the cognitive task at 1 month for all animals. Previous experimental studies in mice revealed a difference at 2-3 months as well as 4-5 months (Dimitrova et al., 2017). We found that the TBI and control animals showed a consistent lack of exploration of both the familiar and novel object as well as showed no object preference, a limitation that is in keeping with Lueptow's (2017) paper on NORT (Lueptow, 2017). Another explanation for the lack of difference between groups on the NORT was explicated by Matsumota et al., (2014) which revealed that rats may have an inherent 'neophobia' for novel object and this may affect performance on NORT. Taken together, we showed that the more sensitive MWM test revealed an impairment in short-term cognition.

4.5 Delayed Brain Dysfunction as Revealed by Electrocorticography

We provided direct electrocorticographic evidence for the occurrence of spontaneous seizures following a modified weight drop model of moderate traumatic brain injury after long-term monitoring of brain activity. We extended our observations by characterizing the seizure phenotype in this model, and to our knowledge, this is the first time for demonstrating spontaneous seizures in a mTBI model. Overall, we showed that

26% of the TBI animals developed PTE between 2-6 months post TBI induction. Experimental findings from previous studies using a fluid percussion injury model of severe TBI reported a 30% (Shultz et al., 2013) and 50% (Kharatishvili et al., 2006) rate of spontaneous recurrent seizures in rats at 6 months post injury induction. To our knowledge, there appears to be no animal study looking at the development of PTE in a moderate model of TBI. The PTE rate that we observed supports findings in human patients which revealed the 24 month cumulative risk of developing PTE following mTBI to be 24% (Englander et al., 2003; Gupta et al., 2014). Klein et al (2018) also reported a congruent finding of 20-50% rates after moderate to severe TBI (Klein et al., 2018). However, there still exists some inconsistencies amongst different studies regarding PTE rates in humans as some studies reported 4.3% (Annengers & Rocca, 1998) and 7.6% (Da Silva & Willmore, 2012). The discrepancy between observations by various animal studies could stem from the methods employed by different studies: biomechanics of injury, age at injury, and lack of universal consensus for seizure definition (Reid et al., 2016). In the current study, we applied a standardized metric using an in-house automated seizure detection software previously shown by our group to be sensitive in detecting epileptic discharges (see methods). We next extended these finding by characterizing the observed seizure phenotype. Interestingly, our result also suggests that the average number of seizures per day increased with time with a mean of about 12 seconds and that 74% of the seizure events occur during the dark phase (active period of the rats) of the light-dark cycle. More so, seizure duration showed an increasing trend, although remained persistently less than 30 seconds. Finally, we demonstrated from Video-ECoG observations that the observed seizure events are likely originating from the frontal-parietal neocortex where our

electrodes were implanted. Our seizure phenotype observation is in accordance with previous studies using the FPI model in rats (Curia et al., 2011; Reid et al., 2016). Reid et al., (2016) showed the average seizure duration for moderate TBI to be between 10-20 s (in 65% of the animals), while Curia et al., (2011) demonstrated that epileptic discharges originated from the frontal cortex, and thus, focal-onset. For the most part, behavioural symptoms such as immobility, facial clonus, and head nodding (Racine 1 and 2) were observed during seizure. Pitkanen et al., (2006) also showed that most seizure events in rats (55%) occur between 7 – 19:00 hr, a finding that is in accordance with our observations. Taken together, these observations comport with the semiology from the clinical evidence of PTE in patients (Gupta et al., 2014).

We next evaluated abnormal cortical activity by performing a power spectrum analysis. The rationale for looking at power spectrum changes was premised upon the fact that slowing of the background ECoG activity as measured by the theta and delta bandwidths is thought to be associated with epilepsy (Milikovsky et al., 2017). Indeed, spectral analysis revealed a slowing of the ECoG in mTBI animals compared to healthy controls. We extended these observations by demonstrating that epileptic animals showed more slowing of activity compared to the non-epileptic counterparts as well as controls. This correlates with findings with human subjects and rodents (Perucca et al., 2013;Tomkins et al., 2011), although, the present study did not replicate a similar difference for delta and alpha bandwidths between the groups. Overall, this observation suggests that circadian rhythm is distorted in epilepsy and epileptogenesis (Milikovsky et al., 2017).

Finally, we sought to describe the relationship between TBI and PSWE- an emerging concept that was first described by our group (Milikovsky et al., accepted). As

previously described, PSWEs are episodic events defined by a median power frequency less than 5 Hz lasting 10 seconds and PSWE might reflect neuronal hypersynchrony. Our observation suggests that TBI animals had more paroxysmal slow wave events per day compared to healthy controls. A significant revelation we found when the TBI animals were further divided into the epileptic and non-epileptic groups was that the epileptic animals appeared to have more PSWE compared to the non-epileptic group, except for a rat who had similar events to the other control and non-epileptic animals. It is notable that we also observed 3 non-epileptic animals with PSWE events of similar magnitude to the epileptic animals. Indeed, a challenge for the near future will be to monitor these 3 animals closely for the development of recurrent seizures. Hence, we interpret this observation as evidence that suggests that PSWE reflects an underlying neuronal hyperexcitability and thus, may be a potential electrocorticographic biomarker during the epileptogenesis process. But first, there is a need to replicate the results of this novel finding by future experiments.

4.6 Blood-Brain Barrier as a Biomarker

Finally, in a bid to find answers to the hypothesis of the current study, we examined the relationship between BBBD and the long-term complications of TBI such as PTE as well as PSWE, and cognitive impairment. The potential of imaging the BBB as a predictor of PTE and its-co-morbidities has been proposed earlier by several studies in animals and humans (Bar-Klein et al., 2017; Pavlovsky et al., 2005; Tomkins et al., 2008; Tomkins et al., 2011). A novel revelation of the current study was that we found a linear relationship between the magnitude of BBBD and memory deficit (Figure 16c). This observation was

consistent with a previous study linking BBB dysfunction to development of cognitive impairment (Wang & Li, 2016). We also showed evidence to suggest that TBI animals showed more BBBD compared to controls. Surprisingly, we did not observe a difference between magnitude of BBB disruption in epileptic animals compared to non-epileptic and control animals. This suggests that BBBD does not appear to be predictor for the development of PTE at 6 months. It is pertinent to reiterate here that our PTE findings suggest that the epileptic discharge were of focal origin, meanwhile, our MRI analysis software evaluated the whole brain pathology. In this line, a direction for future studies will be to perform regional analysis of BBB to evaluate leakage in the frontal and temporal neocortex- the commonly susceptible regions of the brain following TBI. Lastly, we next tested for the relationship between BBBD and PSWE. We found no correlation between the magnitude of BBBD and the average PSWE per day, given our previous observation (Figure 15) where we showed that PSWE may reflect an underlying epileptogenesis.

Some limitations of the current study include low statistical power which stemmed from limited availability of MRI spots. We maximized the available MRI spots, but we only had 22 scans for TBI animals at the 48 hrs time point. Perhaps, greater statistical power could have resulted in an increased positive predictive value for our results. Second, starting from the third month post-TBI induction, some headcaps came off, therefore, these animals could not be monitored and were taken out of the study. Thirdly, given that the transmitter batteries have to be changed under isoflurane anesthesia twice weekly, and it has been established that isoflurane might have a neuroprotective effect as previously established (Statler et al., 1999), this could have decreased the number of epileptic animals detected.

4.7 Conclusion

Here we considered the predictive capability of BBB disruption in the development of the common neurocognitive complications after a moderate traumatic brain injury. We recorded seizures for the first time in a moderate weight drop model of TBI which closely recapitulates human TBI. We further characterized the electrocorticographic dynamics of mTBI animals and found that PSWE and theta frequency bandwidth could reflect epilepsy and epileptogenesis. Finally, we found that BBBD may be a non-invasive potential predictor for the development cognitive deficits. To recap, the current study suggests that BBB imaging may be potentially beneficial to follow up TBI patients and could be a possible therapeutic target for future pre-clinical trials.

4.8 Future Directions

This study revealed that PTE and cognitive disorders are common following a moderate traumatic brain injury. It further revealed that animals with BBB disruption are more likely to develop cognitive deficits. It would be interesting to perform future drug studies to investigate if blockage of the downstream TGF-β signaling cascade that occurs following BBB opening after TBI could prevent the development of PTE and neuropsychiatric complications.

Future studies should examine the brain of the epileptic, non-epileptic and control animals to test the role of neuroinflammation in the development of PTE. Understanding the molecular mechanisms by which TBI results in epilepsy will be beneficial in facilitating development of specific targeted therapeutics during the epileptogenic process.

We also showed that PSWEs may be a potential non-invasive electrocorticographic biomarker of epileptogenesis. It will be interesting to replicate this finding in future experimental studies by increasing the power and evaluating if these findings are consistent with what is observed in human patients with epilepsy.

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