The Role of Inflammation in the Development of Behavioral Changes and Seizure Susceptibility After Traumatic Brain Injury

by

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Abstract

The robust inflammatory response following traumatic brain injury (TBI) can be both beneficial and detrimental. This study directly compares the effects of increasing or decreasing TBI-related inflammation on behavioral outcome and seizure susceptibility. Fluid percussion injury was induced in four groups of young adult male Sprague- Dawley rats: 1) sham injury; 2) TBI; 3) TBI with minocycline; 4) TBI with lipopolysaccharide (LPS). Rats underwent a series of behavioral tests including composite neuroscores, rotarod, novel object recognition and Barnes maze at various time points within the first month post-injury. Approximately nine months after injury, a subset of rats from each group was subjected to a pentylenetetrazol – induced seizure test. Decreasing inflammation by minocycline ameliorated increased seizure susceptibility in injured rats. Upregulating inflammation with LPS did not modify the seizure susceptibility. Both minocycline and LPS revealed mixed effects on behavioral outcomes. Findings suggest a dual role of inflammation after TBI.

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Contributions

Yuqi Lin wrote the thesis, analyzed the data, and conducted the data collection for all behavioral testing, partially seizure threshold test, and partially cytokine concentration measurement. There were contributions by other individuals which are formally acknowledged:

Dr. Aylin Reid (Graduate Supervisor): provided study design, laboratory resources, data analyses, support, mentorship and guidance

Dr. Chiping Wu (Research Associate): conducted surgical operations including the induction of injury and implantation of electrodes; created apparatus for behavioral testing.

Jackie Liu (Research Technician): helped surgical operations, monitored rats, conducted seizure threshold test in part and provided technical support.

Ming Dong Yang (Research Technician): helped measurement of cytokine concentrations, monitored rats, and provided technical support.

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List of Abbreviations

AD	Alzheimer's Disease
ANOVA	Analysis of Variance
AP	Anterior-Posterior
atm	Atmosphere
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BGG	Bovine Gamma Globulin
CBF	Cerebral Blood Flow
CCI	Controlled Cortical Impact
CD68	Cluster of Differentiation 68
CHI	Closed Head Injury
CI	Confidence Interval
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DAI	Diffuse Axonal injury
DAMPs	Damage/Danger-Associated Molecular Pattern Molecules
DC	Detergent Compatible
DV	Dorsal-Ventral
ED	Emergency Department
EEG	Electroencephalography
FPI	Fluid Percussion Injury
GCS	Glasgow Coma Scale
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GH	Growth Hormone
GO	Glasgow Outcome Scale
HMGB1	High Mobility Group Protein B1
Iba1	Ionized Calcium Binding Adapter Molecule 1

ICP	Intracranial Pressure
IFN-γ	Interferon Gama
IGF-1	Insulin-like Growth Factor-1
IL-10	Interleukin-10
IL-10R	Interleukin-10 Receptors
IL-1R1	Type 1 Interleukin-1 Receptor
IL-1R2	Type 2 Interleukin-1 Receptor
IL-1ß	Interleukin-1beta
iNOS	Inducible Nitric Oxide Synthase
I.P.	Intraperitoneal Injection
LFPI	Lateral Fluid Percussion Injury
LOC	Loss of Consciousness
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MFI	Median Fluorescent Intensity
MFPI	Midline Fluid Percussion Injury
MHC	Major Histocompatibility Complex
MinDC	Minimum Detectable Concentration
MINO	Minocycline
MIP-1α	Macrophage Inflammatory Protein -1 alpha
ML	Medial-Lateral
MMP	Matrix Metalloproteinase
MMW	Morris Water Maze
mV	Millivolts
NAC	*N*-acetylcysteine
NF-kB	Nuclear Factor Kappa B
NMDA	*N*-methyl-D-aspartate
NO	Nitric Oxide
NOR	Novel Object Recognition
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PAMPs	Pathogen-Associated Molecular Patterns
PBBI	Penetrating Ballistic-like Brain Injury

PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
PRRs	Pattern Recognition Receptors
psi	Per Pound-Force Per Square Inch
PTA	Post-Traumatic Amnesia
PTE	Post-Traumatic Epilepsy
PTS	Post-Traumatic Seizures
PTZ	Pentylenetetrazole
QCs	Quality Controls
RANTES	Regulated on activation, normal T cell expressed and secreted
rHFOSs	repetitive high-frequency oscillations and spikes
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
RT	Room Temperature
SEM	Standard Error of the Mean
S.Q.	Subcutaneous Injection
TBI	Traumatic Brain Injury
TGF- ß1	Transforming Growth Factor Beta -1
TLR	Toll Like Receptor
TNF- α	Tumor Necrosis Factor – alpha
TNFR1	Tumor Necrosis Factor Receptor Type 1
TNFR2	Tumor Necrosis Factor Receptor Type 2

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1 Literature Review

1.1 Traumatic Brain Injury (TBI)

1.1.1 Epidemiology and Impact

Traumatic brain injury (TBI) is a serious public health issue leading to death and disability (Peterson et al., 2019). In Canada, it is estimated that approximately 155,000 people experience a TBI each year (Rao et al., 2017). In the United States, a recent report by the Center for Disease Control and Prevention revealed approximately 2.87 million TBI-related emergency department (ED) visits, hospitalizations, and deaths in 2014 (Peterson et al., 2019). In Europe, estimates showed that 775,000 individuals sustained a TBI annually (Tagliaferri et al., 2006). However, these figures underestimate the true incidence of TBIs occurring due to various factors, such as exclusion of patients receiving non-emergency care or those who did not seek medical attention (Peterson et al., 2019).

The rates of TBI in different sex and age groups vary. The age groups associated with the highest rates of ED visits, hospitalization and deaths are young children aged 0 to 4 years, individuals aged 15 to 24 years, and seniors aged 75 years or older (Bruns et al., 2003; Peterson et al., 2019; Rutland-Brown et al., 2006; Thompson et al., 2006). Males have a 1.4 times higher incidence of TBI than females across all age groups (Faul et al., 2010). For all age groups, TBI most commonly results from falls, motor accidents, or being struck by an object (Faul et al., 2010; Peterson et al., 2019; Taylor et al., 2017).

TBI can cause substantial healthcare spending, with a total cost of approximately $26.8 million (cost of all injuries including TBI) for Canadians in 2010 (Public Health Agency of Canada, 2018). TBI can significantly change an individual's life, increasing unemployment risk after TBI with the consequences for the family (Doctor et al., 2005; Khan et al., 2003). The burden of TBI represents an essential public health problem that requires more efforts to improve the outcome following TBI.

1.1.2 Classification and Pathophysiology of TBI

Traumatic brain injury is identified as a brain injury caused by an external mechanical force (Maas et al., 2008). Examples are blast injury, a projectile object such as a bullet, acceleration - deceleration forces, or concussive force (Maas et al., 2008). The 15-point Glasgow Coma Scale (GCS) is commonly used to describe the level of consciousness in a patient following TBI and is

usually administered either at the site of the injury incident, or in the emergency department (Andriessen et al., 2010). Injury severity can be classified as 1) mild: a score from 13 to 15; 2) moderate: a score from 9 to 12; and 3) severe: a score of 8 or less (Andriessen et al., 2010; Teasdale & Jennett, 1974). TBI severity can also be classified according to duration of post-traumatic amnesia (PTA), with less than 24 hours of PTA defining mild TBI (Kay et al., 1993), while moderate to severe TBI typically has more than 24 hours of PTA (Malec et al., 2007). Longer duration loss of consciousness (LOC), the period of time from injury until the person regains the capacity to obey commands, is also correlated with higher injury severity (Kay et al., 1993; Malec et al., 2007). Overall, approximately 80% of TBI cases are considered mild, 10% are moderate, and the remaining 10% are classified as severe (Syed et al., 2007). The most common type of TBI, mild TBI or concussion, often shows diffuse axonal injury (DAI) without gross damage in the structure of brain (Kelly & Rosenberg, 1997). DAI is defined as widely spread shearing of the axons, commonly resulting from rapid acceleration and deceleration forces (Gennarelli et al., 1982). Patients with mild TBI may recover within weeks with adequate rest (Centers for Disease Control and Prevention, 2016). Some individuals with mild TBI may show post-concussion symptoms that can include numerous physical, cognitive, and emotional symptoms (Ryan et al., 2003). Post-concussion symptoms such as headache, dizziness, depression, anxiety, and memory problems can last from months to years following TBI (Ryan et al., 2003). Many individuals in contact sports are affected by repetitive mild TBI, that may lead to chronic traumatic encephalopathy accompanied by symptoms such as psychiatric disturbances, difficulty with attention and speech, and impairment in memory and executive functions (Asken et al., 2017).

TBI can be broadly classified as focal or diffuse brain injury (Baethmann et al., 1998; Nortje & Menon, 2004; Werner & Engelhard, 2007). Focal brain damage results from injury types that involve forces impacting on the skull and compressing the underlying brain tissue at the site of impact (coup) or contralateral to the side of impact (contrecoup) (Pudenz & Shelden, 1946). The pathological features and neurological deficits are determined by where the impact to the skull is located and the severity of the impact (Andriessen et al., 2010). Focal brain injury can lead to contusions, epidural haematomas (bleeding in the space between the skull and dura matter), subdural haematomas (bleeding between the dura matter and arachnoid matter), and intraparenchymal haematomas (bleeding in the brain) (Andriessen et al., 2010; National Institute

of Neurological Disorders and Stroke, 2017). Diffuse brain injury is mainly produced by acceleration – deceleration of the head, commonly observed in motor accidents (Gennarelli et al., 1982). Edema and DAI are common clinical characteristics of diffuse brain injury. While the severity of focal injury has not been a successful predictor of poor clinical outcome, DAI has been demonstrated to associate well with cognitive impairments such as memory and executive function after TBI (Sharp et al., 2014). A possible rationale behind such observation is that proper communication between brain networks is required for these higher cognitive functions (Pearn et al., 2017). It is important to acknowledge the possibility of co-existence of both focal and diffuse injuries in a patient with TBI. According to an MRI study by Skandsen and colleagues (2010), focal damages, such as contusions or haematomas, as well as DAI have been reported in 50% of patients with moderate and severe TBI (Skandsen et al., 2010).

Although the GCS helps in the initial assessment of TBI patients, it fails to reveal some essential features of injury or to account for variance in outcome. The underlying pathology cannot be inferred from the GCS. Similar clinical presentations may result from different patterns of structural damage (Andriessen et al., 2010). For example, motor deficits measured by the GCS can be caused by haemorrhage in the basal ganglia or by DAI impacting the corticospinal tract (Jang, 2009). The pathological processes following TBI are complex and include several stages. Direct tissue damage and impairments of cerebral blood flow (CBF) regulation and metabolism are observed in the first stages (Werner & Engelhard, 2007). The reduction of blood flow and oxygen supply results in accumulation of lactic acid, a product of anaerobic glycolysis (Werner & Engelhard, 2007). In addition, the formation of edema can occur (Unterberg et al., 2004). Since cellular energy states cannot be maintained adequately with anaerobic metabolism, the energy-dependent membrane ion pumps fail to function properly (Werner & Engelhard, 2007).

Following the first pathological stages, a depolarization of neurons occurs due to an enormous release of excitatory neurotransmitters, including glutamate and aspartate (Werner & Engelhard, 2007). Activation of *N*-methyl-D-aspartate (NMDA) and voltage-dependent Ca^{2+} and Na^{+} channels leads to increased intracellular levels of Ca^{2+} and Na^{+} (Werner & Engelhard, 2007). Ca^{2+} influx leads to a series of events, including the activation of enzyme caspases, calpain proteins, as well as production of free acids and radicals (Galgano et al., 2017). The build-up of Ca^{2+} caused by glutamate excitotoxicity elevates the mitochondrial production of reactive

oxygen species (ROS), producing further damage (Maciel et al., 2001). In addition to directly damaging neurons, TBI induces a robust inflammatory response and a breach of the blood-brain barrier (BBB) (Galgano et al., 2017; Werner & Engelhard, 2007). Many components of the BBB are vulnerable, including endothelial cells, pericytes, and astrocytes (Shetty et al., 2014). Injury to endothelial cells loosens tight junctions (Shetty et al., 2014) while damaged pericytes and activated astrocytes secrete cytokines, nitric oxide (NO), and matrix metalloproteinases (Pearn et al., 2017; Shetty et al., 2014; Hurtado-Alvarado et al., 2014). Together these factors exacerbate permeability of the BBB, augmenting inflammation cascades (Loane et al., 2014). Such secondary injury mechanisms further induce neuronal cells to undergo necrosis or apoptosis (Werner & Engelhard, 2007).

1.1.3 Inflammatory Response to TBI

1.1.3.1 Overview

Neuroinflammation is a complex response of the brain's immune system to injury or infection (Milatovic et al., 2017). Neuroinflammation after TBI is considered a sterile immune response, because its initiation does not involve a pathogen. (Rock et al., 2009). Various resident cells of the central nervous system (CNS) actively participate in this process (Rock et al., 2009). In addition, peripheral blood-born cells infiltrate the brain after breakdown of the BBB (Jin et al., 2012; Karve et al., 2016). Thus, neuroinflammation involves cellular and secreted factors both within the brain and from the periphery (Helmy et al., 2011b; Thelin et al., 2017). Early inflammatory signalling molecules are secreted within minutes to hours after injury (Gyoneva & Ransohoff, 2015). Damaged resident cells in the brain release intracellular molecular signals, called damage/danger-associated molecular patterns (DAMPs) (Dugue et al., 2017; Gyoneva & Ransohoff, 2015) such as high mobility group protein B1 (HMGB1), S-100 proteins, adenosine triphosphate (ATP), and nucleic acids (Corps et al., 2015). The signaling of DAMPs involves binding with their corresponding pattern recognition receptors (PRRs) that are expressed either at the cell membrane or in the cytoplasm (Dugue et al., 2017; Gorina et al., 2011; Holm et al., 2012). In response to DAMPS-PRR binding, large multiprotein "inflammasome" complexes develop in neurons, microglia, macrophages, and brain endothelial cells (Dugue et al., 2017; Walsh et al., 2014). The inflammasome typically consists of a PRR connected to the protease caspase-1 through an adaptor protein (Dugue et al., 2017; Karve et al., 2016; Lafon et al., 2006;

Goodall et al., 2014). Caspase -1 activation induces the release of cytokines, small proteins that influence cell-cell interaction and augment the neuroinflammatory response (Dugue et al., 2017; Karve et al., 2016; Lafon et al., 2006; Goodall et al., 2014). One example of membrane-bound PRRs is Toll-like receptors (TLR). These include TLR-2 and TLR-4, that have been shown to increase CNS production of cytokines and chemokines (Karve et al., 2016; Lafon et al., 2006; Goodall et al., 2014). The early mediators of damage trigger secondary mechanisms that include 1) activation of glial cells, or "reactive gliosis" (Chiu et al., 2016); 2) further production of cytokines and chemokines; and 3) recruitment of leukocytes from the periphery (Morganti-Kossmann et al., 2001). These processes will be discussed in the following sections.

Interpretation of a neuroinflammatory response is complicated by the fact that both beneficial and detrimental effects on cellular survival are exhibited. Moreover, these effects can change over time. The initial inflammatory response can facilitate repair, while chronic neuroinflammation can contribute to further damage (Chiu et al., 2016; Woodcock & Morganti-Kossmann, 2013).

1.1.3.2 The Response of Glial Cells in TBI

In response to TBI, glial cells become activated in a process called reactive gliosis (Chiu et al., 2016). Changes in the cellular morphology, number and function in microglia and astrocytes alters the interaction between neurons and glial cells as well as between glial cells (Chiu et al., 2016). Characteristics of reactive astrocytes and microglial and their effects on other cells, including oligodendrocytes are discussed in the following sub-sections.

1.1.3.2.1 The Activation of Astrocytes

Astrocytes are the most abundant glial cell type in the CNS (Tower & Young, 1973; Kimelberg & Nedergaard, 2010). In the uninjured brain, astrocytes are essential for maintaining homeostasis within the CNS, with roles of supporting neurons, monitoring the flow of water and K^+, regulating the uptake and release of glutamate and maintaining the BBB (Chen & Swanson, 2003; Tower & Young, 1973). In response to TBI, the reactive process through which astrocytes are activated is called astrogliosis (Sofroniew & Vinters, 2010).

There are many aspects involved in astrogliosis following TBI. The astrocytes undergo morphological alteration and show elevated expression of intermediate filament proteins such as

glial fibrillary acidic protein (GFAP) and vimentin, along with increased production of inflammatory mediators and growth factors (Liu et al., 2013; Gorina et al., 2011; Zamanian et al., 2012; Paintlia et al., 2013). After injury, the morphology of activated astrocytes is characterized by extended processes, hypertrophic swelling of cell bodies, and proliferation of astrocytes toward the damaged tissue (Karve et al., 2016). Hypertrophic astrocytes were observed in injured and surrounding areas three days following experimental TBI (Villapol et al., 2014; Susarla et al., 2014)). At seven days post-injury, ongoing changes in the morphology of astrocytes and the formation of a glial scar were observed (Karve et al., 2016; Villapol et al., 2014). Glial scars commonly form after injury and are made up predominately of astrocytes (Silver & Miller, 2004). The glial scar is an important barrier isolating damaged tissue, thereby preventing harmful molecules and DAMPs from leaking into healthy tissue (Karve et al., 2016). However, glial scars also serve as a barrier to axonal regeneration which can have detrimental effects (Ribotta et al., 2004; Silver & Miller, 2004).

The response of astrocytes after TBI is suggested to play a role in another secondary injury mechanism, excitotoxity, which has the substantial accumulation of extracellular glutamate as a major contributor (Karve et al., 2016). Elevated extracellular glutamate concentration may be related to glutamate transporter dysfunction, as blocking glutamate transporters (EAAT1 and EAAT2) on astrocytes in rats leads to increased level of glutamate (Rothstein et al., 1996). In clinical TBI studies, decreased expression of these glutamate receptors in glial cells has also been demonstrated (Landeghem et al., 2006; Beschorner et al., 2007).

Astrocytes have both protective and detrimental impacts in TBI (Karve et al., 2016). Contradictory results have been reported regarding the roles of activated astrocytes in different experimental models. In one study utilizing a controlled cortical impact (CCI) model in mice, a neuroprotective role of astrocytes was observed, as indicated by increased neuronal degeneration and inflammation following the blockage of proliferating reactive astrocytes (Myer et al., 2006). Another study using fluid percussion injury (FPI) in rats revealed a detrimental impact of astrocytes. In this study, the blockage of proliferating astrocytes resulted in improved cognitive performance, decreased neuronal death, reduced scar formation, and better histological appearance (Di Giovanni et al., 2005). Conflicting findings from these two studies may be attributed to differences in the methods of blocking reactive astrocytes and duration of

such blockage. Ablation of astrocytes in the CCI study was achieved by delivering an antiviral agent, ganciclovir, in a transgenic mouse model (Myer et al., 2006). In contrast, a cell cycle inhibitor, flavoiridol, was used to reduce astrocyte proliferation after FPI (Di Giovanni et al., 2005). Moreover, CCI mice were treated with ganciclovir for seven days immediately after injury, while only one dosage of flavopiridol was administered 30 minutes after FPI (Myer et al., 2006; Di Giovanni et al., 2005).

1.1.3.2.2 Microglial M1/M2 Phenotypes

Microglia are resident immune cells in the brain that are derived from primitive myeloid progenitor cells (Ginhoux et al., 2010; Nayak et al., 2014). Microglia have a variety of functions, such as modulation of synaptic functioning, removal of neurons during development, and cleaning cellular debris through phagocytosis (Nayak et al., 2014; Parkhurst et al., 2013). Microglia can acquire different cellular phenotypes, with heterogeneity of function, depending on the environmental state (Harry, 2013). In response to signals post-TBI, microglia are triggered to undergo morphological change from a ramified or resting state to "M1" or "M2" activated states (Mantovani & Locati, 2009; Sica & Mantovani, 2012). The M1 phenotype can be induced by proinflammatory stimuli such as interferon gamma (IFN- γ) and lipopolysaccharide (LPS) (Lynch, 2009). The M1 type of microglia is believed to be pro-inflammatory and produces pro-inflammatory cytokines, chemokines and inducible nitric oxide synthase (iNOS) (Kigerl et al., 2009). In contrast, the M2 phenotype, which can be induced by exposure to IL-4, IL-13 or IL-10, produces an anti-inflammatory response (Kigerl et al., 2009; Colton, 2009). M2 microglia exhibit increased phagocytic activity *in vitro (*Wang et al., 2013) and release the anti-inflammatory cytokine IL-10 and growth factors such as TGF- ß (Harry et al., 2013; Kigerl et al., 2009).

The dynamic switch between M1 and M2 types largely determines the function of microglia in response to TBI. After injury, microglia migrate to the injured site to remove debris, such as damaged myelin, and start the M1-mediated wound healing process (Harry et al., 2013; Kotter et al., 2011). Although such responses shortly after TBI are regarded as protective, a substantial M1 response can result in aggravation of the injury and persistent tissue damage (Loane et al., 2014). Therefore, a rapid transition to a M2 response is necessary to facilitate repair and neuronal growth (Chiu et al., 2016). One factor, age, has been shown to alter the morphology of

microglia. An increased M1 phenotype has been observed in older populations (Vaughan & Peters, 1974). The age-associated change is accompanied by higher baseline levels of pro-inflammatory cytokines (Harry et al., 2013) and potentially a reduced M2 response (Ye & Johnson, 2001; Fenn et al., 2014; Chiu et al., 2016). Considering age as a risk factor in TBI, detrimental effects can result from elevated levels of pro-inflammatory cytokines and dysfunction in the optimal transition from the M1 to M2 phenotype (Streit et al., 2004). Impairment in the appropriate polarization between these two phenotypes will be detrimental to brain function, especially in the context of TBI.

Studies in preclinical and clinical settings have demonstrated a link between chronic microglial activation post injury and neurodegeneration. Many of these studies identify activated microglia, since various molecules are expressed on the cell surface of microglia (Karve et al., 2016). For example, activated microglia show increased expression of ionized calcium binding adapter molecule 1 (Iba1) (Ito et al., 1998) and cluster of differentiation 68 (CD68) (Graeber et al., 1990). In a human study of TBI *post-mortem* brains, persistent activation of microglia, indicated by higher levels of CD68 and the major histocompatibility complex (MHC) Class II molecule CR3/43, was shown in 28% of cases that survived between 1 year to 18 years after injury (Johnson et al., 2013). Moreover, increased white matter degeneration and myelin breakdown were evident in patients with a chronic inflammatory pattern (Johnson et al., 2013). In a preclinical study of moderate TBI using the CCI model in mice, activated microglia were present for up to 1 year after TBI, detected by increased levels of Iba-1, CD68, and CR3/43 in the ipsilateral cortex (Loane et al., 2014). There were other notable observations including an expanded lesion, white matter pathology, and loss of hippocampal neurons (Loane et al., 2014). The responses of astrocytes and microglia tend to be reciprocal (Karve et al., 2016). Based on the results from studies that modulate the response of astrocytes, microglia activation was increased by depletion of astrocytes, while decreased microglial activation was observed when the activity of astrocytes was excessive (Robel et al., 2011).

1.1.3.2.3 Oligodendrocytes

Although the phenomenon of reactive gliosis predominately involves astrocytes and microglia, it is undisputed that oligodendrocytes are another important glial cell type that play multiple functions in the CNS. A principal function of oligodendrocytes is to form the myelin sheaths

surrounding the axons (Chiu et al., 2016). Additionally, oligodendrocytes can support the survival and growth of cells by producing neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-1 (IGF-1) (Dougherty et al., 2000; Du & Dreyfus, 2002). When there is an insult to the CNS, oligodendrocytes are vulnerable to damage and their dysfunction results in demyelination of white matter tracts (Chiu et al., 2016). Thus, after the injury, the axonal conduction is impaired, which ultimately leads to axonal death (Chiu et al., 2016).

The normal functioning of the CNS cannot be achieved without the coordinated operation of astrocytes, microglial, and oligodendrocytes. Therefore, following TBI, damage to any of these glial cells will result in dysregulation of homeostasis. More importantly, the extent of the TBI-induced gliosis significantly characterizes the influence of neuroinflammation on the outcome after the initial injury.

1.1.3.3 Cytokines as Key mediators after TBI

In addition to the activation of glial cells, the inflammatory response after TBI involves the release of molecular mediators such as cytokines and chemokines (Morganti-Kossmann et al., 2001). Cytokines are small proteins released by immune cells in the peripheral systems as well as microglia, astrocytes and neurons in the brain (Helmy et al., 2011b). Cytokines act as signaling molecules and mediators showing a rapid increase of concentration in response to multiple types of insults to the brain, such as infections, ischemia, and injury (Whalen et al., 2000; Holmin & Hojeberg, 2004; Nazarloo et al., 2003).There are various types of cytokines, including the TNF, lymphokine, IL and IFN families (Dinarello, 2007). The receptors of cytokines can be classified into 1) IL-1 receptor family; 2) TNF receptor family; 3) Class-I or hematopoietin cytokine receptors including receptors of the IL-2, IL-3, and IL-6; 4) Class-II cytokine receptor family including interferon receptors and the IL-10 receptor; and 5) homodimeric receptors (Dinarello, 2007; Ransohoff & Brown, 2012; Shastri et al., 2013). Based on the effect of their actions, cytokines can be broadly described as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines generally begin or perpetuate an inflammatory response, whereas anti-inflammatory cytokines inhibit the inflammatory response (Woodcock & Morganti-Kossmann, 2013). Most cytokines appear to have both opposing roles and they function coordinately with each other in response to the insult (Helmy et al., 2011a). Originally

named “chemotactic chemokines”, chemokines act to attract leukocytes to the injury or infection site (Gyoneva& Ransohoff, 2015). There are four major chemokine groups based on the presence and location of cysteine residues in the structure, including XC, CC, CXC, and CX_3C chemokines, where C and X indicate cysteine and other non-cysteine amino acid residues, respectively (Rollins, 1997). Chemokines act on G protein-coupled receptors and initiate various signaling pathways that eventually result in changes in cell movement along with actin polymerization (Gyoneva & Ransohoff, 2015). There are four families of chemokine receptors, including XLRs, CCRs, CXCRs, and CX_3CRs (Gyoneva & Ransohoff, 2015).

There are four common methods to evaluate the levels of cytokines and chemokines in human studies, including sampling from 1) blood; 2) cerebrospinal fluid (CSF); 3) microdialysis; and 4) direct tissue such as *post-mortem* tissue (Helmy et al., 2011b). Notably, in human studies, most chemokines show higher levels in the CSF as compared to plasma, indicating local production of inflammatory mediators by resident cells in the brain (Helmy et al., 2011a, 2012). The concentrations of cytokines in rodent models of TBI can be detected using the above-mentioned methods and assessed at the level of either mRNA or protein expression (Helmy et al., 2011a). Furthermore, the development of multiplex assays that simultaneously measure multiple mRNA and protein analytes greatly increases the number of inflammatory mediators detected in TBI (Gyoneva & Ransohoff, 2015). Several studies showed increased expression of various cytokines and chemokines, such as IL-1ß and TNFα, IL-6, CCL2, CCL3, CXCL1 and CXCL2 within six hours after TBI (Israelsson et al., 2008; Mukherjee et al., 2011; Dalgard et al., 2012; Shein et al., 2014). Similar observation has been reported in TBI patients, with the peak levels of many cytokines and chemokines within six to 12 hours after TBI (Helmy et al., 2011a, 2012). Timing for maximum level and concentrations of cytokines can greatly depend on the experimental model and injury severity (Helmy et al., 2011b). In the following sub-section, six cytokines (i.e., IL-1ß, TNFα, and IL-10) and chemokines (i.e., MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5) assessed in this current study are discussed in more detail.

1.1.3.3.1 IL-1ß

The pro-inflammatory cytokine, IL-1ß, belongs to the IL-1 family, which is a critical mediator in inflammatory response and elevated shortly after an insult to the CNS as well the peripheral systems (Allan et al., 2005). The IL-1 family also includes IL-1α (an agonist of IL-1ß), IL-1ra

(an antagonist), IL-18, and other members (Dinarello, 1998; Barksby et al., 2007). To be in an active form, IL-1ß precursors must be cleaved by caspase-1(Allan et al., 2005). Several molecules are involved in modulating the transcription and translation of IL-1ß precursors, such as prostaglandins, lipopolysaccharides (LPS), and glucocorticoids (Allan et al., 2005). For example, production of IL-1ß precursors can be increased by LPS, prostaglandins, and intracellular adhesion molecules (Allan et al., 2005). Active IL-1ß binds to two receptors, named the type 1 IL-1 receptor (IL-1R1) and type 2 IL-1 receptor (IL-1R2), while the IL-1R2 is described as a decoy receptor because it does not induce downstream pathways when ligands bind to it (Dinarello et al., 2010). In contrast, complex downstream pathways are induced by the IL-1R1 in many types of cell, such as astrocytes, microglia, neurons, and leukocytes (Allan et al., 2005). One example of the IL-1R1-induced pathway in microglia is to signal the downstream p38 mitogen-activated protein kinase (p38 MAPK) to induce the production of other cytokines and the function of phagocytosis (Dugue et al, 2017; Allan et al., 2005).

There is clinical evidence that IL-1ß concentrations increase after TBI (Frugier et al., 2010; Tasci et al., 2003). In a study evaluating *post-mortem* cortical tissue in patients with TBI, the level of IL-1ß was significantly increased in patients with a survival time between six and 122 hours as compared to the control brains or to patients with survival times under 17 minutes (Frugier et al., 2010). Similarly, in rodent models of TBI, the production of IL-1ß is increased after TBI (Rooker et al., 2006; Knoblach & Faden, 2000). For example, a study using the lateral fluid percussion model (LFPI) reported a significant increase in the level of IL-1ß in the injured cortex at 4 hours after injury (Knoblach & Faden, 2000). An increase in the level of IL-1ß has been found to be linked to aggravated injury and increased cell death (Kamm et al., 2006; Lu et al., 2005). In a weight drop TBI model, hippocampal IL-1ß mRNA and protein expression were increased at three hours, peaked at 12 hours and persisted for up to 48 hours post-TBI (Lu et al., 2005). Administration of IL-1ß antibodies rescued hippocampal neurons post-TBI (Lu et al., 2005).

1.1.3.3.2 TNFα

Although often referred to as a pro-inflammatory cytokine, TNFα produced by microglia and astrocytes displays multiple functions (Woodcock & Morganti-Kossmann, 2013). There are two receptors activated by TNFα, including TNF receptor type 1 (TNFR1) and type 2 (TNFR2).

Both receptors induce complex signaling pathways. Some studies have shown that TNFR1 is typically linked with worsening cell injury as well as exacerbation of cell death, whereas TNFR2 is associated with not only inflammation and apoptosis, but also cell survival and proliferation (Kalliolias & Ivashkiv, 2016; Depuydt et al., 2005).

Increased concentrations of TNFα have been detected following TBI in humans (Morganti-Kossamann et al., 1997; Csuka et al., 1999; Frugier et al., 2010). In a study evaluating *postmortem* human tissues, both ipsilateral and contralateral cortex from TBI patients with six to 122 hours post-TBI survival time had higher level of TNFα than controls and TBI patients with less than 17 minutes survival time (Frugier et al., 2010). Earlier studies have also shown an elevated TNFα level in the CSF and serum of TBI patients compared to controls within post-injury 24 hours (Morganti-Kossamann et al., 1997; Csuka et al., 1999). This increase in TNFα level has been detected in experimental TBI studies, starting at one hour and reaching the peak between four and eight hours following injury (Shohami et al., 1996; Fan et al., 1996; Knoblach et al., 1999). In some studies using the weight-drop and CCI models, the level of TNFα in the CSF and cerebral lysate reached its peak later, at 24 hours post-injury (Hang et al., 2004; Stover et al., 2000).

TNFα appears to have a dual role in the context of TBI. Earlier studies modulating the activity of TNF by its administration or inhibition suggested the increased expression was harmful (Ramilo et al., 1990; Shohami et al., 1997; Trembovler et al., 1999). For example, a study using three agents inhibiting the TNF reported improvement in neurological outcome, along with reduced BBB impairment and edema (Shohami et al., 1997). In contrast, studies using TNF and TNF receptor knockout mice have shown mortality rates were higher and long-term neurological recovery was limited (Scherbel et al., 1999; Stahel et al., 2000).

1.1.3.3.3 IL-10

Predominately seen as an anti-inflammatory cytokine, IL-10 can be generated by neurons, microglia and astrocytes in the CNS and by lymphopoietic cells in the peripheral systems (Aloisi et al., 1999; Mesples et al., 2003; Wu et al., 2005). The receptors for IL-10 are called IL-10 receptors (IL-10R) with two subunits, including IL-10R-α and IL-10R-ß. IL-10 exhibits immunosuppressive effects, such as activating M2 type microglia (Bell- Temin et al., 2015;

Colton, 2009), inhibiting the production of NO and ROS (Csuka et al., 1999), and reducing adhesion of leukocytes to the endothelial cells (Jungi et al., 1994).

In the context of human TBI, it has been shown that the expression of IL-10 is increased in the CSF and serum of patients with severe TBI (Csuka et al., 1999). Some studies have reported elevated IL-10 expression early after severe human TBI, with a peak timing within post-injury two to eight hours (Woiciechowsky et al., 1998). Different temporal profiles of IL-10 expression have been reported in experimental TBI studies. Previously, an increased brain IL-10 level was observed at four hours post injury in the FPI model (Knoblach & Faden, 1998). Intravenous administration of IL-10 in this TBI model improved neurological motor function at post-injury day seven and 14, with decreased expression of TNFα and IL-1ß in the cortex in traumatized rats at post-injury four hours (Knoblach & Faden, 1998). Another study reported a reduction of expression one day after CCI in rats (Lee et al., 2012), while the expression of IL-10 showed no changes over the four days following a weight-drop injury (Yan et al., 2011).

1.1.3.3.4 CCL2 (MCP-1)

CCL2, also known as monocyte chemoattractant protein-1(MCP-1), together with CCR2, play an essential role attracting monocytes out of the bone marrow and recruiting them to injured or infected tissues, including the brain (Chu et al., 2014; Gyoneva& Ransohoff, 2015). The signaling of CCL2/CCR2 has been reported to be involved in TBI. For example, increased levels of CCL2 have been detected in the CSF of severe TBI patients as well as in the injured cortex of traumatized mice, with a peak expression at four to 12 hours after closed head injury (CHI) (Semple et al., 2010). Traumatized mice with CCL2 deficiency in this study have shown reduced lesion size and improved neurological function around one month after injury (Semple et al., 2010). Overall, a beneficial impact in both histological and neurological outcome has been observed when there is deficiency in CCL2 or CCR2 signaling after TBI, suggesting a role of this signaling in TBI pathology (Gynoeva & Ransohoff, 2015).

1.1.3.3.5 CCL3 (MIP-1α)

CCL3, also described as macrophage inflammatory protein -1α (MIP-1α), as well as its receptors, CCR1 and CCR5, have been shown to be expressed by astrocytes, microglia and neurons (Zhu et al., 2012; Xu et al., 2009; Mennicken et al., 2002). The upregulation of CCL3

has been observed in human TBI as well as in experimental models (Helmy et al., 2012; Mukherjee et al., 2011). An upregulated concentration of CCL3 was found by 24 hours post-FPI (Mukherjee et al., 2011). Typically, CCL3 is categorized as a neutrophil chemoattractant (Marciniak et al., 2015). CCL3 has been associated with temporal lobe epilepsy and Alzheimer's disease (Xu et al., 2009; Liu et al., 2014).

1.1.3.3.6 CCL5 (RANTES)

CCL5, also known as regulated on activation, normal T cell expressed and secreted (RANTES), is a chemokine that is responsible for attracting and recruiting immune cells such as T cells, monocytes and basophils (Bachelerie et al., 2014). The signaling of CCL5 can be through three receptors: CCR1, CCR3, or CCR5 (Bachelerie et al., 2014). The mRNA level of CCL5 has been found to be elevated in the cortex after human and animal TBIs (Sandhir et al., 2004; Stefini et al., 2008). In patients with TBI, the upregulation of CCL5 is also observed in their plasma (Helmy et al., 2011a). It is suggested that CCL5 may be involved in modulating pathology in CNS injury. One proposed mechanism is the promotion of T cell recruitment by CCL5 in spinal cord injury, which leads to the production of other chemokines, such as CCL2, to attract monocytes into injured tissues (Jones et al., 2005). Several receptors for CCL5 were expressed both on T cells and monocytes (Bachelerie et al., 2014), resulting in complex signaling and interaction.

1.1.3.4 Recruitment of Leukocytes

The recruitment of leukocytes from peripheral systems contributes to the cascade of neuroinflammation. The population of neutrophils is abundant, and they are frequently the first leukocytes responding to the insult in the peripheral system as well as in the CNS (Kolaczkowska & Kubes, 2013). Studies have shown the maximum number of neutrophils are recruited into the brain approximately one day after the injury (Clark et al., 1994; Rhodes, 2011). By post-injury day three to five the number of neutrophils is largely decreased, while other leukocytes, predominately monocytes, with a limited amount of T cells, natural killer cells and dendritic cells, start to accumulate in the brain (Hausmann et al., 1999; Holmin et al., 1995; Rhodes, 2011). Although the recruitment of neutrophils is important for tissue repair, it can result in breakdown of the BBB through the release of ROS, proteases, metalloproteinases,

TNFα, and metalloproteinases (Corps et al., 2015). Several studies showed no evidence of a link between blockage of neutrophils entering the brain and better neurological outcome (Rhodes, 2011; Weaver et al., 2000; Isaksson et al., 2001).

In contrast to the early infiltration of neutrophils, preclinical and clinical studies show that the peak number of monocyte- derived macrophages does not occur until 24 to 48 hours post-injury (Soares et al., 1995; Semple et al., 2010). The CCL2/CCR2 signaling results in the movement of monocytes into the brain from peripheral systems. Monocytes have the capacity of differentiating into macrophages or dendritic cells (Shi & Pamer, 2011). Studies using CCL2 or CCR2 knockout mice have showed improved functional outcome several weeks following TBI, suggesting a detrimental role of macrophages in the longer term (Semple et al., 2010; Hsieh et al., 2014). The role of T cells is unclear in the context of TBI (Corps et al., 2015). A study conducted on mice lacking mature B and T cells in a CHI model failed to observe a neuroprotective effect for up to one week, suggesting that T cells may not play a role (Weckbach et al., 2012).

1.1.4 Current Treatments for TBI and Challenges

As discussed previously, TBI involves multiple types of injury and a spectrum of injury severity. The choice of treatment strategy depends on many aspects of the situation (Galgano et al., 2017). In more severe forms of TBI, immediate medical care aims at stabilizing the patient's condition and preventing his or her death (National Institute of Neurological Disorders and Stroke, 2017). There are many immediate needs when managing TBI in the acute phase. These include management of intracranial pressure (ICP), optimization of blood flow, and maintenance of ventilation (Abdelmalik et al., 2019). When there is significant impact from a hematoma (e.g., epidural or subdural), or a contusion with a large amount of blood, invasive surgical intervention may need to be performed with the purpose of reducing ICP (Galgano et al., 2017). Databases have been established to promote accurate diagnosis and proper management in the acute phase (Stocchetti & Zanier, 2016). Due to advancement in early treatment and management of TBI patients, the number of individuals surviving acute TBI has increased (Galgano et al., 2017). The longer-term therapeutic interventions after moderate to severe TBI can require various degrees of rehabilitation to promote functional recovery (Khan et al., 2003). There are often two phases of TBI-related rehabilitation. These involve inpatient

management for those with more severe behavioral impairment, followed by community-based management (Khan et al., 2003). Despite available interventions targeting different phases post-TBI, the diverse characteristics of TBI lead to a huge challenge in developing effective treatments (Algattas & Huang, 2014). In the U.S alone, more than 3.5 million individuals suffer from long-term disabilities after TBI (Zaloshnja et al., 2008). It is crucial to develop more effective treatment for improving multiple outcomes after TBI.

An essential factor to consider when developing an effective drug or treatment for TBI patients is having a clinically relevant therapeutic time window (Mohamadpour et al., 2019). A key question is when the treatment can be initiated following TBI. Recent clinical trials have enrolled patients within four to seven hours after a moderate to severe TBI, though most patients are not likely to be treated within such a short window (Mohamadpour et al., 2019). To treat more patients, a drug will need to be effective when patients have the first dosage up to 12 hours after moderate to severe TBI (Mohamadpour et al., 2019). Patients with milder TBI often do not seek medical treatment days after injury until strong symptoms appear (Demakis & Rimland, 2010). In the case of mild TBI, drugs will need even longer therapeutic time windows (Mohamadpour et al., 2019). It is challenging to find the optimal therapeutic time window, due to the complexity and dynamic nature of injury mechanisms following initial injury impact.

During the past 30 years, a variety of clinical trials in TBI have been conducted (Xiong et al., 2015). A review found most acute phase treatment trials did not show an effect or even had an adverse effect on TBI outcomes (Lu et al., 2012). There are currently no drugs to effectively improve neurological recovery after TBI (Xiong et al., 2015). Potential factors contributing to failure in clinical TBI trials include 1) heterogeneity of the TBI patients; 2) variability of patient-specific response; and 3) various clinical characteristics are replicated in some TBI models but not others (Xiong et al., 2015).

Several drugs have shown high therapeutic potential in pre-clinical studies but failed in the clinical setting. For example, progesterone, a female reproductive hormone, has shown neuroprotective effects in preclinical TBI (Melcangi et al., 2014; Xiong et al., 2015). Two Phase III clinical trials failed to find efficacy of progesterone in treating moderate to severe TBI (Wright et al., 2014; Skolnick et al., 2014). In the Phase III PROtect III trial, patients received the first dosage of progesterone within four hours post-injury (Wright et al., 2014). No

significant effect was found in 882 TBI patients regarding their Extended - GOS six months after injury as compared to placebo (Wright et al., 2014). Another study, called SYNAPSE, examined whether progesterone improves the GOS at three and six months after severe TBI. Progesterone did not show any beneficial effect in any of the outcomes when dosed eight hours after injury (Skolnick et al., 2014). Statins, inhibitors of cholesterol biosynthesis used to lower levels of cholesterol, have also been shown to enhance functional recovery in TBI rats (Mahmood et al., 2009). A clinical study of 19 TBI patients and 17 controls failed to show a significant difference in the assessment of amnesia and disorientation when the first dosage of rosuvastatin was given within 24 hours of injury (Sanchez-Aguilar et al., 2013).

1.1.5 Post-Traumatic Mortality and Morbidity

Even if individuals survive the initial injury, TBI can trigger a chronic disease process that results in neurological complications, such as cognitive disturbance, sleep disorders, psychiatric disease, neuroendocrine disorders and seizures (Masel & DeWitt, 2010; Stocchetti & Zanier, 2016). Cognitive impairments typically include deficits of attention, memory, information processing speed, and executive function (Stocchetti & Zanier, 2016). Deficits in sustained attention, paired associative learning, and reaction time were found in patients with moderate to severe TBI, as compared to matched controls (Salmond et al., 2004). In a more recent study, 71 patients with mild to severe TBI were followed up at approximately five years after injury (Marsh et al., 2016). A large range of cognitive dysfunction was observed in many patients, such as impairment in attention, verbal memory, visual memory, and executive functions (Marsh et al., 2016). In addition, complaints of sleep disturbances were common after TBI (Masel & Dewitt, 2010). Among individuals who had sustained TBI, an elevated incidence of obstructive sleep apnea was observed (Castriotta et al., 2007), which is associated with worse cognitive functioning as well as with severe cardiac arrhythmias during sleep (Masel & Dewitt, 2010; Wilde et al., 2007).

Some TBI survivors are affected with psychiatric and psychological deficits as one of the most disabling consequences following initial TBI (Masel & Dewitt, 2010). There is an accumulation of findings linking TBI with an elevated risk of developing multiple psychiatric disorders, such as anxiety disorders, mood disorders, major depression, and obsessive-compulsive disorder (Fleminger, 2008; Masel & Dewitt, 2010; Zasler et al., 2007). Additionally, TBI is associated

with a higher risk of developing substance abuse or dependence (Koponen et al., 2002). A recent meta-analysis has shown that TBI increases the incidence of having bipolar and depression disorders (Zgaljardic et al., 2015). Elevated rates of suicidal ideation (Kishi et al., 2001), attempted suicide (Silver et al., 2001), and completed suicide (Teasdale & Engberg, 2001) have also been linked with TBI.

Neuroendocrine disorders may persist as an outcome of TBI. Approximately 35% of patients with moderate to severe TBI experience chronic impairment of the pituitary axis (Masel & Urban, 2015; Stocchetti & Zanier, 2016). Similarly, another study has reported hypopituitarism in roughly 30% of moderate to severe TBI cases one year post-injury (Schneider et al., 2007). Several hormonal deficiencies have been observed after TBI, including growth hormone (GH), gonadotropin, cortisol, and thyroid (Masel & Urban, 2015). Therapy targeting GH replacement has shown positive outcomes in TBI patients with severe hypopituitarism (Gardner et al., 2015).

TBI has been linked to an elevated risk of dementia (Stocchetti & Zanier, 2016). In a large retrospective study involving more than 50,000 mild, moderate, and severe TBI patients, 8.4% of patients developed dementia (Gardner at al., 2014). The study also found factors such as age and severity of injury could affect the risk of dementia. Moderate to severe TBI was associated with a higher risk of dementia for all ages, while mild TBI seemed to only impact patients aged 65 or greater (Gardner at al., 2014). A more recent study found that a history of TBI was linked to an increased risk of dementia relative to both the general population and to those who experienced non-TBI trauma (Fann et al., 2018). TBI can be a risk factor for multiple neurodegenerative diseases. Numerous pieces of evidence have shown that TBI may be a risk factor for Alzheimer's disease (AD) (Masel & Dewitt, 2010). A large study of World War II Navy and Marine veterans documented that individuals that had sustained TBI had more than twice the risk of developing AD as well as non-AD dementia (Plassman et al., 2000). The severity of the injury appeared to correlate with increase in the risk. The study reported that moderate TBI was linked with a 2.3-fold increase in the risk, whereas severe TBI had a 4.5-fold increase of developing AD (Plassman et al., 2000). Additionally, some studies have shown that TBI is associated with Parkinson's disease (PD). A study of 93 pairs of twins of World War II veterans reported that if both twins developed PD, an earlier onset of PD was more likely seen in the one that had sustained a TBI (Goldman et al., 2006). Another study found that individuals

who suffered from a TBI had an increased risk of PD, and such risk was increased with higher injury severity (Bower et al., 2003).

In addition to neurological complications, a common problem after moderate to severe TBI is musculoskeletal dysfunction that leads to impaired motor functions, which could negatively impact an individual's ability to perform daily activities (Elovic et al., 2004). According to Masel and DeWitt, patients with TBI have about a 30% incidence of bone fractures (Masel & DeWitt, 2010). Bladder and bowl incontinence have been reported in14% of patients with moderate to severe TBI (Safaz et al., 2008). Significant disturbances in sexuality are also reported in the TBI population. A study using self-reports and structured interviews to assess sexual function one-year post-injury found that 29% of subjects complained of sexual dysfunction (Sander et al., 2012). TBI leads to increased mortality compared to general population. Patients with moderate to severe TBI were 2.2 times more likely to die than matched controls, and had an average life expectancy reduction of 6.6 years (Greenwald et al., 2015). In another study, mild TBI had a mortality ratio of 1.33 while moderate to severe TBI had a ratio of 5.29 (Brown et al., 2004).

Significant improvements have been reported in patients in the first six months after TBI (Choi et al., 1994; Pagulayan et al., 2006). In the study by Choi and colleagues, 35% of 786 patients with severe TBI achieved a positive outcome at six months and 5% more patients had the same level at a year post-injury (Choi et al., 1994). More lines of evidence suggest that outcomes following TBI change after longer periods of time, with an additional perspective of considering TBI as a chronic disease process (Stocchetti and Zanier, 2016; Masel & Dewitt, 2010). When being followed-up for 12 to 14 years after the initial TBI, a quarter of patients in a study demonstrated functional impairment between seven and 13 years (McMillan et al., 2012).

1.1.6 Post-traumatic Epilepsy

TBI can cause seizures, which are defined as a transient occurrence of symptoms due to abnormal excessive neuronal discharge in the brain (Fisher et al., 2014). A seizure is distinguished from the term epilepsy, which refers to "a condition in which a person has recurrent seizures due to a chronic or genetically predetermined underlying process" (Marchi et al., 2014). According to the 2014 definition from the International League Against Epilepsy, epilepsy is defined as either: 1) at least two unprovoked seizures that occur more than 24 hours

apart; 2) one unprovoked or reflex seizure and a probability of future seizures similar to the general occurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; or 3) diagnosis of an epilepsy syndrome (Fisher, 2015). Based on the timing of onset following TBI, seizures are categorized as 1) immediate seizures which occur within 24 hours after injury; 2) early seizures which occur between 24 hours to one week after injury; and 3) late seizures which occur more than one week after injury (Frey, 2003). Early post-traumatic seizures (PTS) are considered to be provoked by the head injury (Verellen & Cavazos, 2010) and can result in additional brain damage through elevated intracranial pressure and excess neurotransmitter (Agrawal et al., 2006). Systematic review studies found the antiepileptic drugs phenytoin or carbamazepine protect against early PTS (Beghi, 2003; Chang & Lowenstein., 2003).

Clinically, a diagnosis of post-traumatic epilepsy (PTE) is given when the first late PTS (at least a week post-injury) occurs after TBI (Fisher et al., 2014). Late PTSs are unprovoked, because they reflect permanent instead of temporary changes in the brain (Verellen & Cavazos, 2010). PTE accounts for approximately 5% of all epilepsy in the general population (Hauser et al., 1993). Within two years after the first late seizure, 86% of patients develop a second seizure (Haltiner et al., 1997; Pitkanen et al., 2017). There are several risk factors for the development of PTE in humans (Table 1). For example, the severity of TBI is related to the risk of developing PTE. The 30-year cumulative incidence of epilepsy is increased with more severe injury, with 2.1% for mild TBI, 4.2 % for moderate TBI, and 16.7% for severe TBI (Annegers et al., 1998). According to a large study of 647 participants from multiple centers, 8.0% of TBI patients with a GCS score of 13 to 15, 24.3% with a GCS score of 9 to 12, and 16.8% with a GCS score of 3 to 8 developed late PTS by one year after injury (Englander et al., 2003). Another study reported that the estimated long-term (up to 10 years or longer) relative risk of epilepsy was twice as high after mild TBI, and seven times higher after severe TBI (Christensen et al., 2009). Besides the injury severity, the type of injury also plays a role in the risk of developing PTE. Up to 53% of patients with penetrating brain injuries develop PTE (Frey, 2003; Salazar & Grafman, 2015). Other risk factors for late post-traumatic seizures include older age, early PTSs, skull fracture penetrating the dura, and LOC of more than 24 hours (Pitkänenet al., 2017; Annegers et al., 1998; Englander et al., 2003; Frey, 2003).

Clinical data have not shown any efficacy of antiepileptic drugs in preventing the development of PTE (Temkin, 2009). While being useful in reducing early PTS, phenytoin has been found not effective in the prevention of late PTS (Chang & Lowenstein., 2003). Although 35% of patients with PTE can be effectively treated with antiepileptic drugs alone, the use of polytherapy is required for approximately 60% to 80% of patients (Hudak et al., 2004).

Table 1.1 | Select risk factors for the development of post-traumatic epilepsy. (Annegers et al., 1998; Englander et al., 2003; Frey, 2003; Pitkänenet al., 2017). Abbreviations: LOC (loss of consciousness).

Risk Factors	
More severe injury	Penentrating injury
Older ages	Early post-traumatic seizures
Multiple or bilateral contusions	> 24 h LOC
Acute Intracerebral Hematoma	Skull fracture with dural pentration

1.2 Experimental Models of Traumatic Brain Injury

1.2.1 Overview

In the clinical setting, patients with TBI reveal heterogeneity in pathologies due to numerous injury-related and pre-existing factors, such as the location and severity of the injury as well as pre-existing health status and age (Margulies & Hicks, 2009). The nature of heterogeneity in human TBI makes it challenging to develop a single animal model which reproduces the full spectrum of primary and secondary injury mechanisms observed in the clinical situation (Morales et al., 2005). However, it is essential to utilize animal models, as this allows researchers to investigate complex molecular and cellular mechanisms following human TBI that are otherwise difficult or impossible to be addressed in the clinical setting (Xiong et al., 2013). In the preclinical setting, various conditions including age, gender, genetic conditions and injury parameters can be well controlled, allowing a production of a rather homogenous population to target certain clinical features (Xiong et al., 2013; Johnson et al., 2015). Several species have been used to model TBI, including non-human primates (Ommaya et al., 1966), sheep (Grimmelt et al., 2011), cats (Sullivan et al., 1976), ferrets (Lighthall, 1988), rabbits (Lindgren & Rinder, 1965), and pigs **(**Durham et al., 2000; Zhang et al., 2008). Mice and rats are the most widely used because of cost, smaller size and availability of standardized outcome measurements (Xiong et al., 2013). In the available literature, the classifications of experimental

TBI models are varied. Historically, Denny-Brown and Russel (1941) categorized experimental models into two types: 1) acceleration concussion; and 2) percussion concussion. In the review paper by Cernak and colleagues (2005), experimental TBI models are classified as static or dynamic models, based on the features of the mechanical force used to induce TBI. The static models consist of defined amplitude and duration of the applied mechanical force, focusing on morphological and functional dysfunction (Cernak et al., 2005; David & Aguayo, 1985; Park et al., 1995). One example of a static model is crushing a cranial nerve using forceps for a certain duration of time (David & Aguayo, 1985). Dynamic models result from a mechanical force with defined amplitude, duration, velocity, and/or acceleration (Cernak et al., 2005). Dynamic direct brain injury can be further divided into two types: 1) impact injury; and 2) nonimpact head acceleration injury (Cernak et al., 2005). Blast injury models of dynamic indirect injury have also been designed to replicate human TBI caused by explosives (Cernak et al., 2015; Saljo et al., 2001, 2002a, b).

The impact injury can be induced by four techniques as follows (Kabadi et al., 2010): 1) penetrating; 2) acceleration; 3) direct brain deformation; and 4) CHI. Penetrating models replicates brain injury involving bullets, needles or missiles, which leads to hemorrhage, laceration of tissue, and brain distortion (Carey, 1995). Impact injury can be also induced by acceleration, while the animal's head is either constrained or unconstrained (Cernak et al., 2015; Finnie & Blumbergs, 2002). In rodents, the weight-drop model developed by Marmarou is a common acceleration impact injury (Marmarou et al., 1994; Foda & Marmarou, 1994). To further study the complex sequelae following brain injury, TBI models that induce injury through brain deformation were developed, such as FPI and CCI (Cernak et al., 2005; Finnie & Blumbergs, 2002). Nonpenetration or CHI models have been designed to reflect the characteristics seen in concussive and diffuse brain injury (Cernak et al., 2005; Goldman et al., 1991). Previously, Goldman and coworkers developed a CHI model that strikes the skull at a midline location using a pendulum at a certain angle and force (Goldman et al., 1991). More recently, a new CHI model that modifies the Marmarou weight-drop model has been designed in rats to replicate the frontal impact frequently observed in motor and sports accidents (Kilbourne et al., 2009).

In contrast to the impact injury models, the nonimpact head acceleration models aim to replicate rapid rotation of the brain within the skull instead of a direct impact (Cernak et al., 2005). Such models have been developed in species such as pigs and primates, resulting in diffuse axonal injury (Kabadi et al., 2010; Meythaler et al., 2001).

1.2.2 Commonly Used TBI models

According to a review by Gold et al (2013), the most common TBI models used in the literature are FPI, CCI, and weight drop impact-acceleration. Blast injury (Saljo et al., 2001, 2002a, b) and penetrating ballistic-like brain injury (PBBI) (Williams et al., 2006a, b) have also become popular choices. Each of these TBI models replicates certain clinical features and will be reviewed briefly in the following sections. Since the FPI model is the focus of this project, this model will be discussed in more detail in a separate section.

1.2.2.1 Controlled Cortical Impact Model

The CCI model was first introduced in ferrets (Lighthall, 1988), later with adaptations to be applied in rats (Dixon et al., 1991), mice (Hannay et al., 1999), pigs (Manley et al., 2006) and monkeys (King et al., 2010). Traditionally, the CCI model uses an impact device to deliver mechanical energy onto the exposed intact dura (Gold et al., 2013). Commonly, the device is pneumatic driven, while a newer method uses an electromagnetic device (Osier et al., 2019; Onyszchuk et al., 2007). The CCI model is considered to induce a focal injury, although some diffuse injury occurs (Gold et al., 2013). The CCI model has been associated with contusion, subdural hematoma, subarachnoid hemorrhage, axonal injury, and BBB disruption (Dixon et al., 1991; Lighthall, 1988; Smith et al., 1995; Morales et al., 2005). In many studies using both rats and mice, investigators reported widespread cortical damage and ablation of both gray and white matter (Dixon et al., 1991; Smith et al., 1995). Up to one year after injury, CCI-induced damage in rats, including cortical cell loss and ventricular expansion, has been observed (Dixon et al., 1999). Neuronal cell loss in the ipsilateral hippocampus, dentate gyrus, and thalamus have been reported (Goodman et al., 1994; Hannay et al., 1995), whereas contralateral damage has been also observed with more severe CCI (Smith et al., 1995). Recently, a comprehensive pathological evaluation of the CCI model reported that damage is even more widespread than previously expected, consisting of acute cortical, hippocampal and thalamic degeneration (Hall

et al., 2005). The CCI model possesses high survivability, permitting long-term outcomes following TBI to be investigated (Morales et al., 2005). Behavioral assessment has revealed that many aspects of functional outcomes have been compromised after CCI, including motor, cognitive and emotional deficits. Motor deficits following CCI in rodents have been observed including but not limited to, loss of both forelimb and hindlimb reflex up to 15 days (Nakamura et al., 1999), and impairment in rotarod task at 48 weeks post injury (Shear et al., 2004). Cognitive deficits assessed in the Morris Water Maze (MWM) task have been reported up to 11 months in the rat CCI model (Verbois et al., 2003). The CCI model causes depressive-like and anxiety behaviors measured in tasks such as the forced swim test and elevated plus maze (Kochanek et al., 2002).

Compared to many other TBI models, the strength of the CCI model is the high reproducibility, because the impact parameters, such as time, velocity and depth of the mechanical force can be precisely controlled (Cernak, 2005). In the review by Xiong and colleagues, the lack of risk of a rebound injury adds another advantage of this model, as compared to models that use a device driven by the force of gravity (Xiong et al., 2013). While the CCI model was originally designed as an invasive model, the model has been applied to study closed head injury, including repeated concussions associated with many sports (Petraglia et al., 2014). More deficits have been observed after repetitive versus single TBI, including tasks of cognition, memory, and sleep (Petraglia et al., 2014).

1.2.2.2 Weight Drop Impact-Acceleration Models

In weight drop models, the injury is induced by a weight falling freely to the exposed skull with, or without, prior craniotomy (Morales et al., 2005). Mechanical parameters can be altered, including the mass of the weight and the height from which it falls, in order to modulate different injury severity (Xiong et al., 2013).

There are several versions of weight drop models, each of which has various characteristics (Xiong et al., 2013). In an early model developed by Feeney and colleagues (1981), the intact dura is exposed, and the weight is dropped to impact the dura through a craniotomy, resulting in cortical contusion. The contused cortex progresses to develop a cavity by 24 hours, and the cavitation appears to be ongoing over two weeks (Dail et al., 1981). Most functional outcomes

recovered by two weeks post injury (Feeney et al., 1981). However, persistent deficits have been reported beyond three months with severe contusions (Gasparovic et al., 2001).

In 1988, Shohami and colleagues developed a CHI model that entailed placing the rodent's head on a hard surface and delivering the impact to one side of the exposed skull (Shohami et al., 1988). Based on work by Shohami's group, a mouse CHI model was established, in which a classic weight-drop device was used to produce focal injury (Flierl et al., 2009). In this mouse CHI model, many clinical characteristics of TBI have been replicated, including neurological impairment, breach of BBB, activation of glial cells, and neurodegeneration (Xiong et al., 2013).

Another popular weight drop model is Marmarou's impact acceleration model, designed to mimic DAI caused by falls or motor accidents (Marmarou et al., 1994). In Marmarou's weight drop model, the skull of a rat is exposed by a midline incision, followed by the mounting of a stainless-steel disc to the skull midline between lambda and bregma, so as to prevent skull fracture (Xiong et al., 2013). The impact is delivered to the exposed skull by a brass weight hitting the disc. The Marmarou model has been reported to induce widespread and bilateral damage along with broad DAI in vulnerable areas, such as the corpus callosum, internal capsule, and the long tracts in the brainstem (Foda & Marmarou, 1994). Motor and cognitive deficits, such as beam walking and memory impairments, have been reported with this model (Marmarou et al., 1994; Schmidt et al., 2000)

More recently, a modification of the Marmarou model, referred to as the Maryland model, has been developed to study CHI. The Maryland model involves delivery of impact to the anterior part of the cranium, resulting in rotational acceleration of the brain inside the intact cranium (Kilbourne et al., 2009). The injury leads to reduced spontaneous exploration that lasts for more than one week after TBI (Kilbourne et al., 2009). More efforts will be still needed to characterize this model (Kilbourne et al., 2009).

Noteworthy strengths of weight-drop models are the low cost, procedural simplicity, minimal invasiveness, and capacity of recapitulating graded DAI as seen in human TBI (Gold et al., 2013; Xiong et al., 2013).

1.2.2.3 Models of Blast TBI

Animal models of blast TBI have been designed to study the effects of blast waves, as may be experienced on the battlefield (Warden, 2006). A blast model involves the use of a long metal tube, closed at one end (Xiong et al., 2013). Rodents are placed at the open end of the tube, while an air pressure wave or an explosion is delivered at the closed end of the tube (Cernak et al., 2001).

Long and colleagues used a compression-driven shock tube to induce blast injury and evaluate the effect of a Kevlar vest that protected the thorax and part of the abdomen (Long et al., 2009). The Kevlar vest significantly reduced mortality in rats and rescued the abundant axonal fiber degeneration, indicating that the blast causes TBI at least partially through systemic effects, such as hypotension and hypoxemia (Long et al., 2009; Xiong et al., 2013). While the blast injury model involves non direct impact, it has been found that even a low level of blast exposure increases intracranial pressure and leads to cognitive impairment in rats (Saljo et al., 2009). A report found persistent deficits in social recognition, spatial memory and motor coordination in mice subjected to mild blast brain injury (Koliatsos et al., 2011).

Increasing evidence suggests that blast injury models induce brain pathology, mechanisms, and biomarkers, which differ from those seen in other TBI models that have focal mechanical components (Bhattacharjee, 2008; Xiong et al., 2013). Neuropathologic features associated with blast models include phosphorylated tauopathy, chronic neuroinflammation and neurodegeneration, without signs of macroscopic tissue damage or hemorrhage (Goldstein et al., 2012; Xiong et al., 2013). While the functional problems are the major health issues observed in the clinical setting, a review has pointed out that blast models have been predominantly focused on tissue damage rather than functional deficits (Xiong et al., 2013). Further research is needed to investigate the long-term functional outcomes (Xiong et al., 2013).

1.2.2.4 Penetrating Ballistic-like Brain Injury Models

In human TBI, PBBI is commonly caused by a bullet wound (Gold et al., 2013). Williams and colleagues characterize PBBI as a high energy injury transmitted by projectiles, which results in the formation of a temporary cavity in the brain, usually having a size of the projectile itself (Williams et al., 2006 a, b). The PBBI model induces injury by the insertion of a specially

designed probe into the brain at a defined location, and an attached balloon is then quickly inflated to resemble the temporary cavity caused by a penetrating bullet (Gold et al., 2013; Williams et al., 2005).

In rats, a PBBI model that focuses on the unilateral frontal brain has been identified to cause the formation of brain edema, BBB permeability, motor (rotarod and balance beam tests) and cognitive (spatial learning in the MWM test) impairment (Shear et al., 2010, 2011). Comparable to other TBI models, the PBBI model produces features that include hemispheric swelling, increased ICP, white matter injury remote to the injury site, and neuroinflammation (Williams et al., 2006 a,b,2007). One distinct characteristic of the PBBI model is that penetration and the formation of the temporary cavity lead to large-scale intracerebral hemorrhage throughout the initial lesion (Xiong et al., 2013).

1.2.3 Fluid Percussion Injury (FPI) Models

1.2.3.1 Model Overview

FPI model is one of the earliest described and best characterized animal models of TBI (Osier et al., 2015). FPI model was initially described in 1965 by Lindgren and Rinder (1965) in rabbits, followed by the use in cats (Sullivan et al., 1976). Eventually, Dixon and colleagues (1987) described the midline FPI model in rats, and McIntosh et al. (1989) modified the craniotomy position away from the midline to establish the lateral FPI model. Adaptation of the FPI model has also been made for pigs (Pfenninger et al., 1989; Zink et al., 1993) and mice (Carbonell et al., 1998) to study TBI.

The process of FPI involves drilling a hole in the rat's skull to expose the intact dura and a plastic cap is implanted over the craniotomy (Gold et al., 2013). The FPI device contains a cylinder filled with fluid which is attached to the plastic cap through a Luer-Loc connection (Gold et al., 2013). The induction of FPI is via a fluid pressure pulse hitting the exposed dura, which is generated by the release of a weighted pendulum striking the piston at the other end of the tube (Thompson et al., 2005). An external pressure detector connected to the FPI device measures the delivered fluid pressure (Thompson et al., 2005). The height at which the pendulum is released is the only mechanical parameter that can be adjusted in a standard FPI devices (Xiong et al., 2013). Recently, the FPI device has been advanced with the use of

computers to precisely control several key features of the fluid percussion waveforms, promoting the reliability and reproducibility of the model (Wahab et al., 2015). There are three sub-types of FPI models, including midline (MFPI), parasagittal, and lateral (LFPI) models based on the relative position of craniotomy to sagittal suture (Ma et al., 2019). When the craniotomy is centered at the sagittal suture, it is called MFPI (Ziebell et al., 2016). Parasagittal FPI has the craniotomy located less than 3.5 cm lateral to midline (Floyd et al., 2002; Sanders et al., 1999). In LFPI, the craniotomy is located 3.5 cm or more lateral to midline (Vink et al., 2001).

1.2.3.2 Pathophysiological Outcomes

The fluid percussion results in brain deformation and displacement, while the strength of the pressure impulse determines the severity of injury (McIntosh et al., 1989). The FPI model replicates both focal and diffuse human TBI without skull fracture (Thompson et al., 2005). This model replicates several important features of clinical TBI, including intracranial hemorrhage, brain swelling, gray matter damage, and DAI (Graham et al., 2000; Thompson et al., 2005). The fluid percussion induces various primary injury mechanisms, consisting of contusion, shearing or stretching of tissue and subdural hematoma (Alder et al., 2011). This is followed by secondary injury mechanisms involving complex changes in molecular, biochemical, and cellular aspects (Thompson et al., 2005). Different FPI models result in distinct histological changes. The LFPI model produces damage predominantly in the injured side (ipsilateral) of the brain, allowing comparison of damage in ipsilateral and contralateral (non-injured side) sides of the brain (Ma et al., 2019). For midline and parasagittal FPI models, both ipsilateral and contralateral cortical changes are shown, which is believed to be linked with direct axial movement of the lower brainstem (Cernak, 2005). Milder injury is typically shown in MFPI model as compared to lateral and parasagittal FPI models. Producing a more severe injury with MFPI is limited because there is a substantially increased mortality due to prolonged apnea resulting from brainstem compression in this model (Cernak, 2005).

There are both short-term and long-term pathophysiological outcomes associated with FPI. Occurring within minutes of the impact, LFPI induces both focal cortical contusion and diffuse subcortical neuronal injury (Hicks et al., 1996). By 12 hours after injury, this combination of injury further progresses to neuronal loss, without expanding to other brain regions by post-

injury day seven (Hicks et al., 1996). Bramlett and Dietrich (2002) discovered that a cavity is generated at the injury site due to the enlargement of the contused cortex over weeks and continuous cell death contributes to its expansion up to one year after injury (Bramlett and Dietrich, 2002). In an earlier rat study, tissue loss was found to start within one hour after the injury and to persist for up to one year after parasagittal FPI (Smith et al., 1997). This study reported prolonged changes in the hippocampus, such as shrinkage of the layers of the pyramidal cells and neuronal loss in the dentate hilus at one year post-injury (Smith et al., 1997). In addition to damage in the cortex and hippocampus, other ipsilateral brain regions are vulnerable to progressive neurodegeneration that starts early after injury and persists over months. These regions include the thalamus, striatum and amygdala (Hicks et al., 1996; Liu et al., 2010; Thompson et al., 2005).

1.2.3.3 Behavioral Outcomes

Human TBI is considered a complex disease process rather than a single event (Masel & DeWitt, 2010), since both primary and secondary injury can lead to detrimental effects on structure and function (Davis, 2000). It has been shown that motor and cognitive impairments can be caused by both focal and diffuse injury (Morales et al., 2005). Assessing behavioral function following experimental TBI is crucial to understand the outcome in human injury (Morales et al., 2005). In comparison to other larger animal models, one outstanding advantage of rodent models is the ready availability of behavioral tests to measure different functional outcomes (Gold et al., 2013). In TBI rodent models, these include behavioral tasks measuring strength, return of reflexes, vestibulomotor and cognitive functions (Fujimoto et al., 2004).

Tests of reflexes reflect the loss of reflexes seen after human TBI and correlate well with outcome measures in the clinical setting, especially the motor aspects of the GCS (Tesdale et al., 1974; Dixon et al., 1987). Common tests include the return of righting reflex, pinna reflex, and cornea reflex. The return of righting reflex is defined as the latency to regain an upright position after the injury impact (Fujimoto et al., 2004). The pinna reflex is indicated by a head shake when the auditory nerve is touched and the cornea reflex is indicated by an eye blink in response to touching the cornea gently with cotton (Fujimoto et al., 2004). These tests of reflexes have been useful to indicate the severity of LFPI (Hallam et al., 2004; Thompson et al., 2005).

Sensorimotor function can be assessed by a battery of motor tests. The composite neuroscore (NS) is established based on many commonly used tests in human patients, such as the Glasgow Outcome Scale (GOS) and Disability Rating Scale (Fujimoto et al., 2004). Laboratories vary in the specific components and scale of the scores for the composite neuroscore testing. In an early version by McIntosh and associates (1989), the composite neuroscore is the sum of four individual motor scores, including 1) forelimb flexion; 2) hindlimb flexion; 3) resistance to bilateral repulsion; and 4) ability to stand on an angle board (Fujimoto et al., 2004). In an early study by Pierce and colleagues (1998), a worse composite neuroscore was reported in severe FPI up to two months after the injury. Another study using composite neurological scores revealed neurological impairment after mild and moderate FPI compared with the sham controls, up to three weeks after injury (Kabadi et al., 2010). In severe FPI, the deficit was observed up to 12 weeks after injury (Zhang et al., 2005).

Vestibulomotor tests used to reveal important daily functions include balance, coordination, and walking. These typically involve fine and complex motor function rather than simple tests of reflex (Fujimoto et al., 2004). Training prior to injury is necessary for animals to learn and acquire such tests. Commonly used vestibulomotor tests are the beam balance, beam walk, rotarod (RR) and rotating pole tests (Fujimoto et al., 2004). Studies using these tests have shown deficits in vestibulomotor function ranging from days to weeks after LFPI (Thompson et al., 2005; Doll et al., 2009; Riess et al., 2007).).

Experimental TBI studies often use spatial mazes or operant boxes to measure cognitive outcome, such as Morris Water maze, Barnes maze (BM), passive avoidance tests, and radial arm maze (Fujimoto et al., 2004; Maegele et al., 2005; Lee et al., 2013). For example, TBI models including FPI have been shown to cause impaired performance in a novel object recognition (NOR) task (Huang et al., 2014). Both anterograde amnesia (learning) and retrograde amnesia (memory) have been found to be impaired after LFPI (Thompson et al., 2005).

In summary, studies have demonstrated that LPFI produces a variety of neurobehavioral deficits that are observed in human TBI, such as suppression of reflexes, difficulties in vestibuomotor function, and cognitive dysfunction such as learning and memory (Morales et al., 2005; Hamm,

2001). In the case of severe LFPI, neurological dysfunction and cognitive impairment have been shown to last for more than a year (Pierce et al., 1998).

1.2.3.4 Induction of Post-Traumatic Epilepsy

The FPI model has been frequently applied to induce PTE (Brady et al., 2018). The targeted strategies aimed at preventing PTE can be developed by identifying biomarkers and mechanisms of post-traumatic epileptogenesis. For example, one potential biomarker of PTE was repetitive high-frequency oscillations and spikes (rHFOSs) (Bragin et al., 2016; Reid et al., 2016). For example, one study reported rHFOSs in 61% LFPI rats versus 14% sham rats (Reid et al., 2016). In addition, the occurrence of rHFOSs was significantly higher in moderate or severe TBI rats compared to rats with mild injury (Reid et al., 2016). Moreover, inflammation is suggested to be one of the potential mechanisms contributing to the epileptogensis after TBI (Missault et al., 2019; Semple et al., 2017). This mechanism is thus the focus of the current study.

The occurrence of spontaneous seizures in injured rats has been described in the FPI model. One study found spontaneous focal seizures that started anterior to or at the injury site occurred in 50% of LFPI rats (Reid et al., 2016). In a severe LFPI model, a study found that 43% to 50% of injured rats developed epilepsy within a 12-month follow up period (Kharatishvili et al., 2006). In another study, 30% of the LFPI rats showed spontaneous recurring seizures at 6 months after TBI (Shultz et al., 2013).

Many TBI studies examined the injured animals' susceptibility to seizure induced by pentylenetetrazol (PTZ). PTZ, as a $GABA_A$ receptor antagonist, exhibits significant convulsant feature in rodents (Veliskova et al., 2017). A previous study of parasagittal fluid-percussion injury found increased susceptibility to PTZ-induced seizures at 2 weeks after FPI (Bao et al., 2011). Similarly, another study conducted by Atkins and colleagues reported increased seizure susceptibility in parasagittal FPI rats at post-injury 12 weeks (Atkins et al., 2010). The Kharatishvili group found increased seizure susceptibility even at 12 months post-LFPI (Kharatishvili et al., 2006).

1.3 Inflammation-modulated Strategies

Currently, there are no treatments to improve outcomes after TBI. Delayed secondary injury mechanisms provide a promising therapeutic window for developing potential treatments. In this study, we focus on one specific secondary injury mechanism, inflammation. The inflammation induced by experimental TBI models has been thought to be a mediator of post-injury outcomes, such as contusion volume, microglial response, behavioral recovery, and post-injury kindling epileptogenesis (Kokiko-Cochran et al., 2018). However, there are conflicting findings of the post-injury consequences with the use of pro-inflammatory or anti-inflammatory strategies. The following sections will discuss studies using bacterial endotoxin LPS and anti-inflammatory drug, minocycline (MINO) in the context of experimental TBI studies.

1.3.1 Lipopolysaccharide (LPS) Studies

LPS, from the outer membrane of Gram-negative bacteria, stimulates macrophage and microglia produced proinflammatory factors, such as TNF-α, IL-1ß, nitric oxide, and prostaglandin E2 (Marsh et al., 2009; Eslami et al., 2015). LPS acts on Toll-like receptor 4 (TLR4) and initiates signaling pathways through nuclear factor kappa B (NF-kB)-dependent and NF-kB-independent pathways (Palsson-McDermott & O'Neill, 2004). To evaluate how inflammatory challenges prior to or subsequent to the induction of TBI might alter the outcome from TBI, TBI studies have utilized LPS as either pre-injury (preconditioning) or post-injury peripheral immune challenge. In both instances, the administration of LPS altered post-injury outcome.

Preconditioning animals with low-dose LPS has been found to be neuroprotective to a subsequent CNS insult, such as stroke and spinal cord injury (Eslami et al., 2015). The same phenomenon has been studied in experimental TBI (Longhi et al., 2011; Eslami et al., 2015). In a previous study, a single injection of LPS (0.1mg/kg) 5 days before the induction of CCI decreased the production of CD68 (phagocytic microglia/macrophage) and elevated the secretion of IL-6 in injured mice, which was associated with improvement of behavioral function and reduced contusion (Longhi et al., 2011). In a more recent study, a single dosage of LPS (0.1 or 0.5 mg/kg) 5 days prior to CCI induction was found to delay post-injury kindling epileptogenesis in rats (Eslami et al., 2015). In this study, pre-injury LPS injection rescued neuronal loss and decreased the overexpression of IL-1ß and TNFα in the hippocampus (Eslami

et al., 2015). Together, these studies using LPS preconditioning showed positive effects on various outcome following TBI.

There are also studies using LPS as a peripheral immune challenge after TBI (Collins-Praino et al., 2018; Fenn et al., 2014; Muccigrosso et al., 2016). Worse outcomes have been observed in these studies. A study induced midline FPI in adult mice and challenged the injured mice with peripheral LPS at 30 days following injury (Fenn et al., 2014). This immune challenge exaggerated the response of microglia by increasing the expression of major histocompatibility complex (MHC-II), IL-1ß, and TNFα compared to the TBI-only and sham control groups (Fenn et al., 2014). A follow-up study confirmed that LPS challenge (0.33 mg/kg) at 30 days post-injury augmented memory recall deficits in injured mice (Muccigrosso et al., 2016). Another study reported depressive-like behavior and cognitive impairments three months after the injury are associated with a magnified inflammatory response induced by LPS five days following TBI (Collins-Praino et al., 2018). These studies showed subsequent post-injury immune challenge with LPS elicits an exaggerated inflammation response and behavioral deficits.

1.3.2 Minocycline (MINO) Studies

Minocycline is a tetracycline antibiotic that can penetrate the BBB (Garrido-Mesa et al., 2013). In addition to its antibiotic properties, minocycline has anti-inflammatory activity (Garrido-Mesa et al., 2013). Several studies have found minocycline has the capability to limit neuroinflammation after TBI (Abdel Baki et al., 2010; Adembri et al., 2014; Haber et al., 2013; Homsi et al., 2009; Sanchez Mejia et al., 2001; Siopi et al., 2011, 2012). Minocycline treatment has been shown to improve performance on various behavioral tasks. For instance, a study showed minocycline decreased locomotor hyperactivity in CHI mice at post-injury day two (Homsi et al., 2010). One group reported minocycline treatment in a focal CHI model was able to improve neurological recovery from three days post-injury which was sustained over six weeks (Ng et al., 2012). In another study, rotarod performance at post-injury days two to four after TBI was significantly improved by minocycline treatment (Sanchez Mejia et al., 2001). In addition to beneficial effect on motor function, minocycline attenuated impaired performance in NOR task at four- and 13-weeks following CHI injury in mice (Siopi et al., 2012). Moreover, minocycline treatment significantly improved the performance on Barnes maze spatial learning

in mild blast injured rats at both short-term (eight days after injury) and long-term (45 days after injury) (Kovesdi et al.,2012).

Minocycline has been shown to exhibit anti-inflammatory properties by inhibiting microglia activation (Vonder Haar et al., 2014; Siopi et al., 2011; Haber et al., 2013). One group has suggested that the inhibitory effect of minocycline on microglia is possibly through reducing production of IL-1ß (Homsi et al., 2010). Furthermore, minocycline has been demonstrated to prevent both gray matter and white matter injury (Abdel Baki et al., 2010; Siopi et al., 2011; Sanchez Mejia et al., 2001).

Previous studies using minocycline have not principally focused on seizure susceptibility and the development of PTE. A small experimental molecule, named Minozac, was used to examine the seizure susceptibility induced by electroconvulsive shock (Chrzaszcz et al., 2010). Minozac was similar to minocycline, acting as an inhibitor of proinflammatory cytokine production and demonstrated reduced seizure susceptibility one week after a midline closed head injury in mice (Chrzaszcz et al., 2010).

Overall, many studies display the importance of inflammation on the outcome of TBI, with a lack of adequate studies investigating the impact on PTE. Inflammation has been linked to other types of epilepsy (Maroso et al., 2010; Vezzani et al., 2011). For example, the expression of HMGB1 and TLR were increased in chronic epileptic mice as well as human epileptogenic tissues, while antagonists of these two reduced acute and chronic seizures (Maroso et al., 2010). Furthermore, there is increasing evidence of an association between inflammation and the development of PTE (Diamond et al., 2014; Semple et al., 2017; Missault et al., 2019). In a clinical study, higher CSF/serum IL-1ß ratios were associated with an increased risk for PTE over time in adults with moderate-to-severe TBI (Diamond et al., 2014). A study using the CCI model found higher subacute and chronic seizure thresholds when treating injured postnatal 21 days mice with IL-1Ra (Semple et al., 2017). Therefore, it is reasonable to assume inflammation after TBI is linked to PTE.

2 Evaluating the Role of Inflammation after TBI in Behavioral Changes and Seizure Susceptibility

2.1 Rationale and Aims

In Canada alone, there are an estimated 155,000 TBIs every year (Rao et al., 2017). Rather than being an isolated and acute injury, TBI leads to an ongoing process that impacts multiple body systems (Masel & Dewitt, 2010). Many individuals who sustained TBI have demonstrated functional impairment following the initial TBI (McMillan et al., 2012). Impairments are frequently observed in motor function (Elovic et al., 2004), coordination and balance (Fork et al., 2005), and cognitive function such as learning and memory (Stocchetti & Zanier, 2016). Furthermore, post-traumatic epilepsy (PTE) is another deleterious consequence after TBI, accounting for approximately 5% of all epilepsy in the general population (Hauser et al., 1993). Importantly, there is currently no effective treatment to prevent the development of PTE (Temkin, 2009).

There are primary and secondary injuries associated with TBI. Acutely at the time of injury, primary damage occurs resulting from the mechanical damage (Werner & Engelhard, 2007). (Werner & Engelhard, 2007). Seconds to minutes following primary injury, secondary injury is triggered and consists of complex cellular and molecular processes that usually cause secondary cell death and extended neurodegeneration (Schimmel et al., 2017; Kabadi & Faden, 2014). Secondary injury can have prolonged impact from days to years following initial injury (Kabadi & Faden, 2014; Werner & Engelhard, 2007). Among various secondary injuries, neuroinflammation is a major secondary injury mechanism in response to the TBI (Helmy et al., 2011a, b). There is increasing evidence of an association between inflammation and the development of PTE (Diamond et al., 2014; Semple et al., 2017; Missault et al., 2019). Since it is not possible to reverse the primary damage, targeting the secondary injury might take advantage of a therapeutic window to improve outcomes following TBI (Schimmel et al., 2017).

The role of neuroinflammation following TBI appears to have a duality with both detrimental and beneficial impact (Woodcock & Morganti-Kossmann, 2013). This is evident by conflicting results reported in previous TBI studies with pro-inflammatory (peripheral LPS immune challenge) and anti-inflammatory strategies (minocycline treatment). While previous literature examined the impact of inflammation on behavioral outcome, the impact of inflammation on seizure susceptibility after TBI has been rarely addressed. In order to have a better

understanding of the role of inflammation on multiple outcomes following TBI, it is important to directly compare the effect of upregulating and downregulating TBI-induced inflammation.

In the current study, we sought to address these information gaps in a rat model of FPI using standardized tests of rodent behaviors as well as direct measurement of CNS cytokine levels. We aimed to characterize the FPI model on inflammatory response, behavioral outcome, and seizure susceptibility. We also sought to modulate the inflammatory environment with agents to reduce inflammation (MINO) and to exacerbate inflammation (LPS).

Our specific aims were 1) to investigate the impact of inflammation-modulating drugs on the expression of CNS cytokines and chemokines following TBI; 2) to investigate the impact of inflammation-modulating drugs on motor and cognitive functions following TBI and 3) to investigate the impact of inflammation-modulating drugs on seizure susceptibility following TBI.

2.2 Hypotheses

1) Neuroinflammation contributes to worse behavioural outcomes after TBI. Administration of LPS 30 minutes before TBI will lead to more severe motor and cognitive deficits, while daily administration of MINO will lead to milder deficits

2) Neuroinflammation contributes to neuronal hyperexcitability and increased seizure susceptibility after TBI. Administration of LPS 30 minutes before TBI will further increase seizure susceptibility, while daily administration of MINO will decrease seizure susceptibility.

This study induced TBI in young adult male Sprague-Dawley rats using the FPI model. One group of animals was administered 100 ug/ml LPS 30 minutes before FPI to exacerbate acute inflammatory responses. A separate group was treated daily with 45 mg/kg MINO following TBI to decrease inflammation. To the test the first hypothesis, a series of behavioral tests were performed at various time points within the first month post-injury. Composite neuroscore and rotarod were performed to assess motor functions, while NOR and the Barnes maze tested hippocampal-dependent memory functions. To test the second hypothesis, animals was injected

with 30 mg/kg PTZ and electroencephalography (EEG) was recorded for one hour to assess seizure susceptibility.

3 Methods

3.1 Ethics Statement

All animal procedures were approved by the University Health Network (UHN) Animal Care Committees and conducted in accordance with the policies and guidelines formulated by the Provincial Government of Ontario and the Canadian Council on Animal Care.

3.2 Animals

Adult male Sprague-Dawley rats weighing between 200g and 250g were purchased from Charles River Laboratories, Canada. Upon arrival, rats were housed in individual cages in groups of two in a room controlled for temperature (20°C to 22°C) and humidity (45%) Furthermore, the room was on an alternating 12-hour light-dark cycle and the animals were provided with *ad libitum* access to food and water. The animals were handled for two minutes daily for at least six days to acclimate to the laboratory setting before any experimental procedures.

3.3 Study Design

This study consisted of two experiments. The study design and experimental timeline are illustrated in Figure 3.1. The number of animals in each experiment is indicated in Table 3.1. The first experiment (Exp. 1) aimed to investigate the impact of inflammation on behavioral outcomes and seizure susceptibility following FPI. Within one month after brain injury, animals in this study were subjected to a battery of behavioral tests, including composite neuroscore, rotarod, novel object recognition, and Barnes maze. Rats were randomly assigned to one of the following four experimental groups: 1) Sham: rats that underwent craniotomy without FPI; 2) TBI-only: rats that underwent FPI only; 3) TBI + LPS: rats that were injected with LPS prior to FPI to upregulate the inflammatory response; and 4) rats that underwent FPI and were treated with MINO to reduce the inflammatory response after injury. Approximately four to six months following injury, the rats were implanted with intracranial electrodes for long-term video-EEG monitoring (results not part of this thesis work).

A subset of 32 rats (n = 8 per experimental group) was subjected to a pentylenetetrazol (PTZ) seizure susceptibility test approximately nine months following injury. To further examine the results of cognitive behavioral testing that were found initially, 12 naïve rats performed NOR and BM testing. Naïve animals were sacrificed four days after completion of behavioral testing and brain tissues were collected.

The second experiment (Exp. 2) aimed to examine the temporal profile of the inflammatory response shortly after brain injury to determine whether the effects seen with pharmacological modulation of inflammation were related to the expression of cytokines and chemokines. A total of 50 rats were sacrificed at either six hours following injury (Exp.2.a) or post-injury day seven (Exp.2.b). The first time point was chosen to investigate the acute inflammatory response after TBI, because increased expression of many CNS cytokines was reported within six hours after experimental TBI (Israelsson et al., 2008; Mukherjee et al., 2011; Dalgard et al., 2012; Shein et al., 2014). The latter time point was selected to capture the delayed inflammatory response. TBI studies reported increased levels of cytokines such as MCP-1/CCL2 and MIP-1α/CCL3 at post-injury seven days (Dalgard et al., 2012; Otto et al., 2001). These acute and subacute time points also allowed us to examine inflammation-regulated changes over time after TBI. For example, these time points involved different numbers of minocycline injection (two doses at six hours following injury versus eight at post-injury day seven), which potentially influenced the inflammatory response at a specific time point.

Comparable to animals in Exp. 1, Exp. 2 also consisted of four experimental groups: 1) Sham (n = 4 for each time point); 2) TBI-only (n = 5 or 6 for each time point); 3) TBI + LPS (n = 7 for each time point); and 4) TBI + MINO (n = 6 or 7 for each time point). Composite neuroscore testing was conducted on all animals. Specifically, all animals performed baseline testing, while the rats sacrificed on post-injury day seven (Exp. 2.b) were tested again two days following injury.

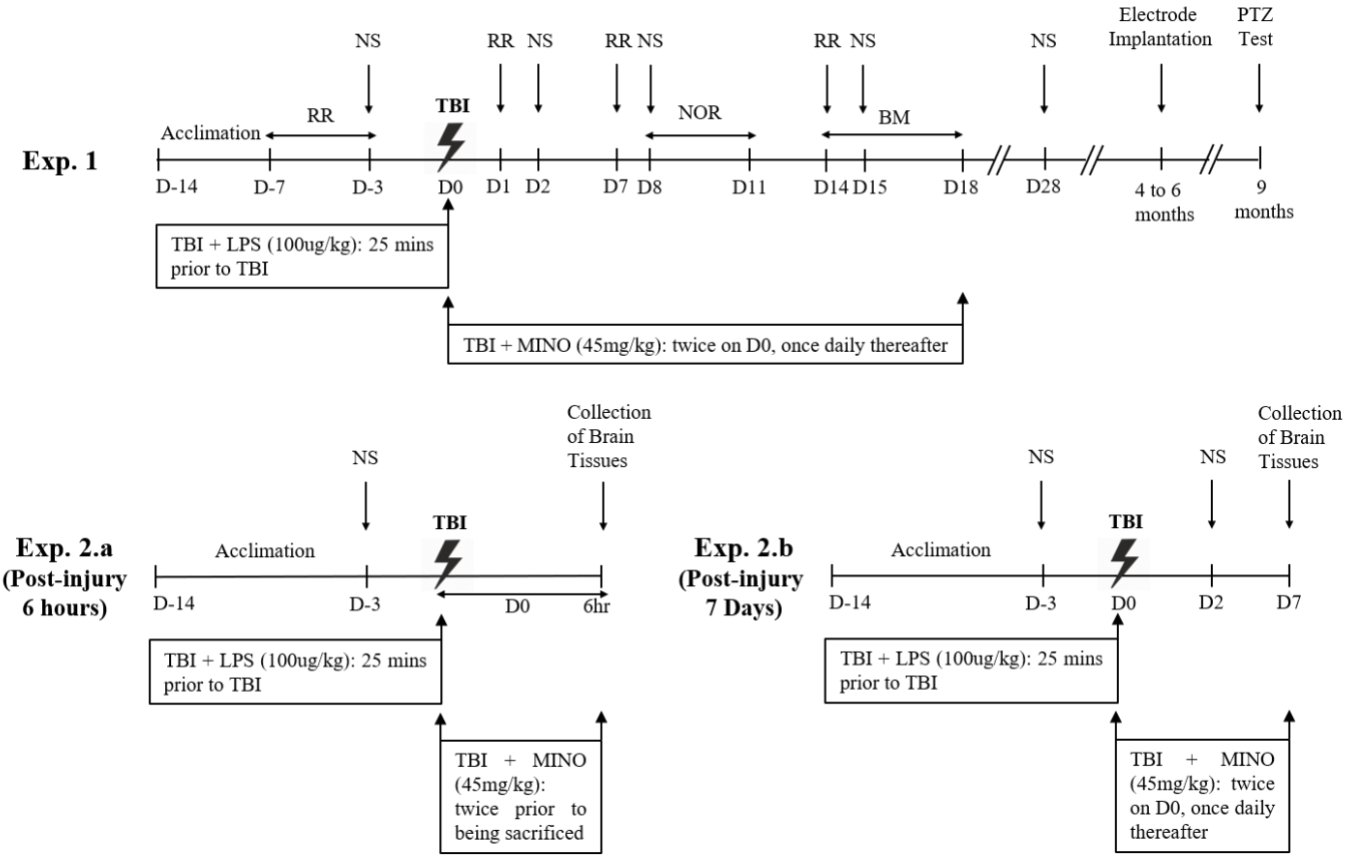

Figure 3.1 | Study design and experimental timeline. Abbreviations: BM (Barnes maze), LPS (lipopolysaccharides), MINO (minocycline), NOR (novel object recognition), NS (neuroscore), PTZ (pentylenetetrazol), RR (rotarod), TBI (traumatic brain injury).

Table 3.1 | Number of animals in each experiment. n/a indicates not applicable.

	Naive	Sham	TBI-only	TBI + LPS	TBI + MINO
Experiment 1	N =12	N = 13	N = 14	N = 17	N = 18
Experiment 2a	n/a	N = 4	N = 5	N = 7	N = 7
Experiment 2b	n/a	N = 4	N = 6	N = 7	N = 7

3.4 Preparation of Drugs

Both MINO (minocycline hydrochloride, catalog # M9511-1G) and LPS (lipopolysaccharide from *E.coli*, catalog # L8274-10mg*)* were purchased from Sigma-Aldrich. The MINO solution was prepared by dissolving the powder in sterile 0.9% NaCl. The solution was heated at 95°C and vortexed intermittently, until it turned a clear yellow color. Fresh MINO solution was made on the day of injection in a concentration of 10 mg/ml. An LPS stock solution at a concentration

of 5 mg/ml was prepared by dissolving the powder in sterile 0.9% NaCl and stored at -20°C. On the day of injection, fresh 100 ug/ml LPS solution was diluted from the stock solution.

3.5 Induction of FPI

3.5.1 TBI-only and Sham Groups

The surgical procedure and the induction of FPI in the TBI-only group were the same for animals in both experiments #1 and 2 (Fig 2.1). The rat was placed in a chamber and anesthetized with 5% isoflurane. Once the rat was unresponsive, its head was shaved and it was placed in a stereotaxic frame with 1.5% - 3% isoflurane via a nose mask. An appropriate level of isoflurane was maintained by closely monitoring the toe pinch response, respiration patterns and body color. The rat was lying on a heated pad to maintain normal body temperature. A midline incision was made in the scalp to expose the skull. Then, a 5 mm diameter craniotomy centered 4.5 mm posterior and 2.5 mm lateral to bregma, was made over the left cortex with a Dremel hand drill without penetrating the dura. This craniotomy location was chosen based on previous studies showing damage to cortex, hippocampus and subcortical layers in the ipsilateral hemisphere as well as to the contralateral hemisphere (Vink et al., 2001). Deficits in neuromotor function, sensorimotor coordination, and spatial reference memory have been shown in the FPI model when using this craniotomy location (Doll et al., 2009). In addition, this specific craniotomy location has been used to study PTE induced by FPI in recent studies (Casillas-Espinosa et al., 2019; Ndode-Ekane et al., 2019; Santana-Gomez et al., 2019). A plastic injury cap (5 mm diameter) was prepared prior to the injury by cutting the female Luer-lock off of a 16-gauge needle. The unused injury caps were kept in 95% alcohol and wiped dry before using. Figure 3.2 shows the process of making the injury cap**.**

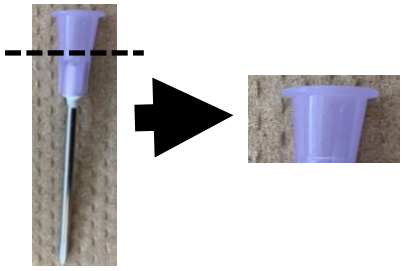

Figure 3.2 | Representation of making a plastic injury cap from a needle. The female Luer-lock was cut off from a 16-gauge needle to prepare a plastic injury cap (5 mm diameter) prior to injury.

The plastic injury cap was affixed into the craniotomy hole using glue and dental cement, taking care not to puncture the dura. The injury cap was filled with saline once it was dried and secured. The rat was then taken off from the frame, discontinuing the anesthesia, and the injury cap was connected to the fluid percussion injury device. The rat's toe pinch response was closely monitored to ensure that the rat was injured under the light influence of anesthesia due to its certain neuroprotective effects (Statler et al., 2000; Statler et al., 2006). Once the rat showed the first sign of responsiveness, TBI was induced with a fluid percussion injury device (Model 01-B, Custom design and Fabrication, Richmond, VA). This FPI device consists of a fluid cylinder (63 cm in length and 6 cm in diameter) filled with degassed water. A piston was arranged to slide within the cylinder. A pendulum, set to fall an angle between 13 to 13.5 degrees was released to strike the piston, compressing the fluid toward the end that was connected to a trauma inducer pressure transducer amplifier. The pressure transducer, with an output of 10 millivolts (mV) per pound-force per square inch (psi), was connected to a digital storage oscilloscope (Tektronix, TDS 1002). Once the pendulum hit the piston, a wave of fluid pressure from the degassed water in the tube of the injury device was applied to the exposed dura through the contact of the saline inside the injury cap. Once the impact was delivered to the rat, the fluid pressure was measured by the pressure transducer and stored in the oscilloscope in mV, which was converted to psi and atmosphere (atm). Figure 3.3 illustrates the FPI device and its components.

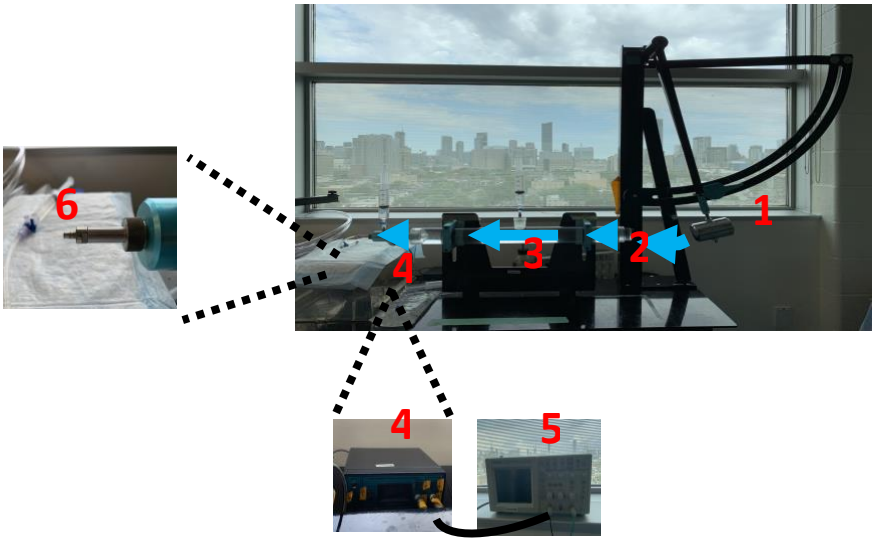

Figure 3.3 | An overview of the fluid percussion injury device and its components: the pendulum (1) is released to strike a piston (2) that pushes liquid in the cylinder (3), producing a pressure that is recorded by the external pressure transducer (4) connected with an oscilloscope (5). The liquid was pushed out through the nozzle (6) that connected with the injury cap of the rat.

The duration of apnea was recorded from the time of impact to the time when the rat produced first spontaneous breathing. The rat continued to be closely monitored by removing it from the injury device and placing it in a left lateral recumbent position on a heated pad. The time of righting reflex was assessed, which was defined as the duration of regaining an up-right position on four limbs after the time of injury. The righting reflex has been shown to be correlated with the severity of injury (Hamm, 2001). Once the rat restored the righting reflex, it was placed back in the stereotaxic frame under anesthesia (1.5% - 3% isoflurane) for removal of the injury cap and closure of the scalp (3-0 ETHILON suture). Saline (3% of body weight) was injected subcutaneously (s.q.) and the rat was placed back to the home cage once it was awakened. Twenty-four hours following the injury, the rats were injected once with meloxicam (10 mg/kg, s.q.) and saline (approximately 3 ml, s.q.). To monitor post-operative conditions, signs of normal behavioral such has grooming were regularly checked and the weight was measured each day for one week after the TBI. KMR® Kitten Milk Replacer Powder was provided to help post-operative recovery. Sham animals in both experiments #1 and 2 were anesthetized and underwent the surgical procedure of craniotomy to expose the intact dura without being subjected to FPI.

3.5.2 TBI + LPS group

The TBI + LPS animals in both experiments were given a single dose of LPS (100 μg/kg) through intraperitoneal injection (i.p.) prior to the induction of the injury. This low dosage of LPS was chosen because it has been used in studies evaluating the effect of preconditioning with LPS pre-injury injection (Longhi et al., 2011; Eslami et al., 2015) as well as experiments with post-injury immune challenge using LPS (Collins-Praino et al., 2018; Corrigan et al., 2017). Systemic injection of LPS at this dosage (i.p.) in mice has been reported to result in increased concentration of cytokines in the plasma, such as elevated TNFα expression at post-injection 1 hour (Murray et al., 2012). LPS was administered before the midline incision, which was approximately 30 minutes before the rat was connected to the FPI device. The remaining FPI procedure for the TBI + LPS rats was the same as the TBI-only rats.

3.5.3 TBI + MINO group

The TBI + MINO animals in both experiments were subjected to FPI and assessed for duration of apnea and righting reflex as described above.

On the injury day (D0), the TBI + MINO rats in experiment #1 received MINO twice. The first dose of MINO (45 mg/kg i.p.) was administered after the rat regained righting reflex. Approximately 4 to 5 hours following injury, the rats were subjected to a second dose of MINO (45 mg/kg i.p.). For the next eighteen days after injury, the TBI + MINO rats in experiment # 1 received MINO (45 mg/kg i.p.) once daily. This dosage of MINO has shown neuroprotective effects in many animal models of TBI (Homsi et al., 2009; Sanchez Mejia et al., 2001). Improved performance on various behavioral tasks have been reported in studies when minocycline was dosed between five minutes and one hour after the injury (Abdel Baki et al., 2010; Haber et al., 2013). Although repeated administrations of minocycline typically occur every 12 hours, we reduced the interval between the first and second injections to target acute inflammation, similar to a previous study (Homsi et al., 2009), as the half-life of minocycline is approximately two to three hours (Andes & Craig, 2002; Colovic & Caccia, 2003). The chronic minocycline treatment paradigm was determined based on evidence showing persistent activation of microglial for 16 days or more after the induction of CCI in rats (Lam et al., 2013). The TBI + MINO rats in experiment # 2 were subjected to the injection of MINO (45 mg/kg i.p.) twice on the injury day as those in experiment # 1. The TBI + MINO rats sacrificed on

post-injury day seven also received the once-daily injection of MINO (45 mg/kg i.p.) for seven days after the injury day. Furthermore, to minimize possible effects of injection on the behavior, MINO was administered at least 30 minutes before the rats performed any behavioral testing.

3.6 Assessment of Behavioral Functions

A series of behavioral tests that measure distinct functions were conducted in this study, including 1) motor function: composite neuroscore; 2) vestibulomotor function: rotarod; 3) recognition memory: novel object recognition (NOR); and 4) spatial learning and memory: Barnes Maze. The behavioral testing was conducted by an experimenter blind to the experimental condition of each rat. All the behavioral testing was done during the light cycle between 9 am and 5 pm. The rotarod and Barnes Maze testing were conducted in the morning, while the NOR and composite neuroscore testing were in the afternoon. For animals performing more than one behavioral test on the same day, the order of testing is shown in Table 3.2.

Table 3.2 | Order of multiple behavioral testing on a day.

Post-injury	Order of Multiple Behavioral Testing
Day 8	1) NOR; 2) Composite Neuroscore
Day 14	1) Barnes maze; 2) Rotarod
Day 15	1) Barnes maze; 2) Composite Neuroscore

3.6.1 Composite Neuroscore

The Composite Neuroscore measures motor function as an evaluation of FPI severity and recovery (McIntosh et al., 1989; Pierce et al., 1998; Niskanen et al., 2013; Hayward et al., 2010). The testing protocol was adapted from previous studies (Kharatishvili, Sierra, Immonen, Gröhn, & Pitkänen, 2009; Febinger, Thomasy, & Gemma, 2016). The testing for experiment #1 was conducted at baseline (pre-injury day 3 or 4) and post-injury day two, eight, 15 and 28. Animals in experiment #2 performed baseline composite neuroscore, while rats sacrificed on post-injury day seven were subjected to composite neuroscore testing on post-injury day two again.

The assessment of a composite neuroscore was conducted on a table (114 cm length x 76 cm width x 69 cm height). An individual score from 0 (severely impaired) to 4 (normal) was given to each of the following seven indices: 1) and 2) left and right forelimb flexion when the tail was suspended; 3) and 4) left and right hindlimb flexion when the tail was lifted up and back while the hindlimb remained on the surface of the table; 5) and 6) the strength to resist both left and right lateral pulsion; and 7) the ability to stand on an angle board in vertical, left and right positions. A composite neuroscore ranging from 0 to 28 was obtained by combining all seven individual scores. The specific scoring for indices will be explained in detail in the following sections.

3.6.1.1 Forelimb Flexion Test

The rat was gently lifted by grasping the base of the tail and its head was slowly lowered toward the flat surface of the table. The motions of both right and left forelimbs were observed for 10 seconds while the procedure was repeated. A score for each forelimb was given as follows: Score 4 = forelimbs were extended forward to reach the surface, away from its body and toes spread; Score 3 = forelimbs were briefly retracted toward the abdomen for less than 2.5 seconds and toes spread; Score 2 = forelimbs were retracted toward the abdomen for between 2.5 to 5 seconds with curled toes; Score 1 = forelimbs were retracted toward the abdomen for more than 5 seconds with curled toes; Score 0 = nose hit the surface first while forelimbs were retracted, and toes curled for the entire 10 seconds being lifted. The table was cleaned with 70% ethanol after testing each animal.

3.6.1.2 Hindlimb Flexion Test

The rat was gently pulled up and back by its tail while the forelimbs remained on the flat surface. Both right and left hindlimb responses were monitored for 10 seconds. A score for each hindlimb was given as follows: Score 4 = limbs extended outward and away from its body, and toes spread; Score 3 = limbs were retracted toward the body for less than 2.5 seconds, and toes spread; Score 2 = limbs were retracted toward the body for between 2.5 to 5 seconds with curled toes; Score 1 = limbs were retracted toward the body for more than 5 seconds with curled toes; Score 0 = limbs were retracted toward the body, and toes curled for the entire time. The table was cleaned with 70% ethanol after each testing.

3.6.1.3 Lateral Pulsion Test

The rat was placed on a disposable absorption pad (56 x 56 cm) on the table, facing away from the experimenter. Lateral pressure with the use of a metal chopstick was applied to the rat's midsection to determine its ability to resist the pulsion. The animal was pushed to the right first and then to the left. A score was obtained for each side as follows: Score 4 = the rat showed complete resistance to falling and it grasped the mat strongly without taking any step away from the pressure source; Score 3 = the rat demonstrated moderate to strong resistance and one step was taken away from the pressure source; Score 2 = the rat exhibited moderate resistance and took more than one steps away; Score 1 = the rat showed slight resistance, but it eventually rolled over; Score 0 = the rat had no resistance, and it rolled over easily. The chopstick was cleaned with 70% ethanol after each rat and the pad was disposed once it was dirty.

3.6.1.4 Angle Board Test

Lastly, the angle board test measured the maximum angle the rat was able to stand on an inclined board after injury relative to the baseline angle. The surface of an angle board (30 x 45.5 cm) was made of a vertically grooved rubber mat. The board was able to achieve an angle between 25° to 60° in increments of 2.5°. The angle board was cleaned with 70% ethanol after each testing and a new trial started once it was dried.

During baseline assessment, the angle board was first set at 35°. The rat was first placed in a vertical direction, followed by left and then right. In order to be considered as a successful completion of a tested angle, the animal needed to stand still on the board for 5 seconds without sliding down. Once every direction was attempted in a set angle, the angle was increased by 2.5°. The angle kept increasing until the rat was not able to stand on the board in any of the directions. The maximum angle at which the animal was able to maintain each position was recorded as a baseline value and given a maximal score 4.

On post-injury day two, eight, 15, and 28, the starting angle at each position was 10° below each rat's baseline angle. A score was assigned to each position compared to the baseline angle as follows: Score 4 = no difference or higher than the baseline; Score 3 = 2.5° decrease from the baseline; Score 2 = 5° decrease from the baseline; Score 1 = 7.5° decrease from the baseline;

Score 0 = 10° or more decrease from the baseline. The final score for the angle board testing was determined by averaging three scores.

3.6.2 Rotarod

The rotarod test is a sensitive task to measure motor coordination in rats after FPI (Hamm, 2001). The protocol was adapted from previous FPI studies (Hamm, 2001; Day et al., 2017). The rotarod device (LE8505 model, Panlab Harvard Apparatus, Spain) consisted of a 60-mm diameter central rod with a knurled surface (Figure 3.4). The rod was separated into four slots which allowed up to four rats to be tested at the same time. To further prevent interference between animals in neighboring lanes, an extension hood was placed above the slot separator. The device was equipped with a timer in each lane to automatically calculate the latency for the rat to fall off from the rotating rod. The switch for each timer was associated with a lever plate underneath each slot. The timer was switched on when lifting the corresponding lever. Once the corresponding lever was pressed down by the falling of the rat, the timer ended. The latency to fall off in seconds for each rat was automatically generated and recorded in an excel sheet in the SEDECOM software (Panlab Harvard Apparatus, Spain). At the beginning of each trial, the rat was placed on the stationary rod facing away from the experimenter. Once all the rats were seated on the rod properly, the rod started rotating at a speed of four rotations per minute (rpm) and the rotational speed was steadily increased to a maximum speed of 40 rpm over an interval of 120 seconds. The termination of each trial was determined when either the rat fell off from the rotating rod or the total 120 seconds elapsed, whichever came first. During pre-injury day 14 to seven, animals in experiment #1 were trained with three trials each day. The interval between trials was approximately five minutes to allow resting for the rats. Three days before the induction of FPI, the baseline latency to fall off for each rat was measured over three trials. Following the injury, the rats were subjected to the rotarod testing on post-injury days one, seven, and 14. The average of three trials for each testing day was calculated for each rat. After each testing, the device was cleaned with 70% ethanol to remove odors between rats.

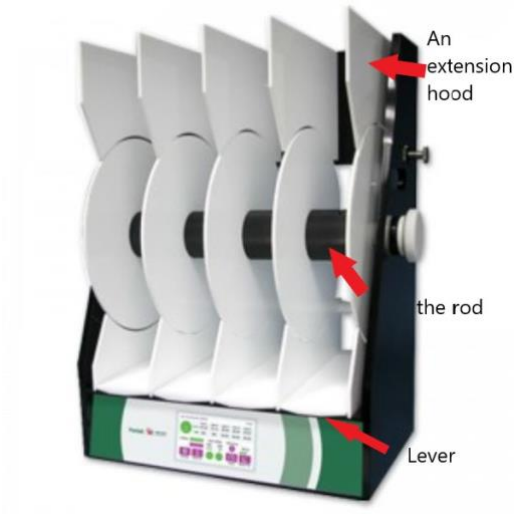

Figure 3.4 | The rotarod machine used in the experiment. The rotarod test was used to measure motor coordination after FPI. A rat was placed on the stationary rod that started rotating from four rotations per minute (rpm) to a maximal speed of 40 rpm over an interval of 120 seconds. To further prevent interference between animals in neighboring lanes, an extension hood was placed above the slot separator. The device was equipped with a timer in each lane to automatically calculate the latency for the rat to fall off from the rotating rod. The switch for each timer was associated with a lever plate underneath each slot. Figure adapted from: https://www.panlab.com/en/products/rotarod.

3.6.3 Novel Object Recognition

Novel object recognition test was used to test hippocampal-dependent recognition memory, which was first outlined by Ennaceur and Delacour (1988). The NOR testing was based on the rat's natural tendency to explore a novel object (Bevins & Besheer, 2006). Our protocol was established from a study by Reger and colleagues (2009). The NOR testing chamber (70 x 70 x 30 cm) was made of blue acrylic (6mm thickness) and placed on top of a flat surface at a height of 90 cm, shown in Figure 3.6. The objects used for the testing included several household items made of glass and metal materials shown in the Figure 3.5.

Figure 3.5 | Objects used in the novel object recognition test.

Like the previous study (Reger et al., 2009), objects were selected that did not consist of eyespots or resemble any animal shape. There were five objects in total and each object has two identical copies. The choices of the location and new objects were randomly assigned to each rat. Each object was secured to the chamber floor using Velcro and placed in the center near the back wall. An object was positioned 12 cm from the back wall and 18 cm from the other object. The NOR testing consisted of a habituation phase and a testing phase. Animals in experiment #1 conducted three habituation sessions from post-injury days eight to10. A testing phase including a familiar trial and a test trial was performed on post-injury day 11 with an interval time of one hour. Naïve rats performed the NOR testing from 21 to 24 days after their arrival, which was equivalent to the timing in other animal groups in experiment #1. During the habituation phase, the rat was allowed to explore the empty chamber without objects for 10 minutes each day for three consecutive days. Twenty-four hours after the last habituation session, two identical objects were placed in the chamber as to-be-familiarized objects (Figure 3.6 A). During this familiar trial, the rat was released from the mid-point of the wall opposite to the back wall with its nose pointing away from the objects to prevent bias. The rat stayed in the chamber for object exposure for three minutes. One hour after the familiar trial, a testing trial was conducted by replacing one familiar object with a new object (Figure 3.6 B). During the testing trial, the rat was released in the same way as the familiar trial and explored the chamber for three minutes.

A circular zone was digitally drawn around each object, which was 2 cm away from its edges (Figure 3.6 C). The SMART software (Panlab Harvard Apparatus) automatically tracked the movement of the rat and measured the duration of time interacting with each object, which was identified when the rat's nose entered this circular zone indicating an exploration behavior. The time interacting with each object during familiar and testing trials was recorded. The video footage of each trial was carefully reviewed by an experimenter blinded to the condition of the rat. If the rat was simply sitting on the edge of the zone, grooming inside the circle, climbing on the object without direct nose contact, or quickly passing through the area without the direct interaction, such detection was considered as a tracking error. The time of incorrect tracking was subtracted from the total interaction time. The objects and chamber were cleaned with 70% ethanol to remove any odors between rats.

A) **B)** **C)**

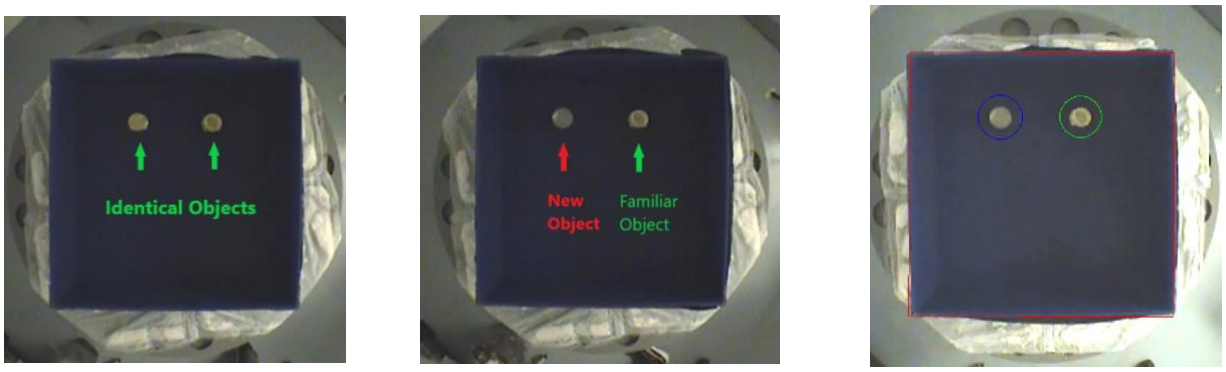

Figure 3.6 | The apparatus and set up for NOR testing. A) During a familiar trial, two identical objects were placed to the back of the wall and the rat stayed in the chamber for object exploration for three minutes; B) During a testing trial, one familiar object was replaced with one new object and the rat stayed in the chamber for object exploration for three minutes; C) A digital circle was drawn around each object, which was 2 cm away from its edges.

3.6.4 Barnes Maze

The Barnes Maze was used to test hippocampal-dependent spatial learning and memory after injury (Barnes,1979). Aversive stimuli such as bright lights were used in Barnes maze testing room to motivate the rats to enter a target box which provided a darker environment. The maze was a grey circular platform with a diameter of 120 cm (Figure 3.7.A). Along the perimeter of the maze, there were 20 holes covered by either a regular chamber or a target box that contained a bigger space for the rat to escape from bright lights and hide inside. There were 19 regular chambers with a diameter of 10 cm and a depth of 2 cm. The target escape box with a depth of 10 cm had a rectangular shape (35.5 cm height x 11 cm inner width). The platform was elevated 90 cm away from the ground. To record trials, a camera (Sony) was mounted approximately 132 cm above the maze on a tripod that was 248 cm away from the floor. Two 150W floodlights (Model # OFTM 300Q 120 LP BZ M6, Lithonia Lighting) were placed about 135 cm above the maze. Two 65W LED bright white lights (Model # BR30, Luminus Elite LED) were placed at the two ends of the tripod around 165 cm away from the ground and 120 cm away from the edges of the maze. Four colored shapes including a square, triangle, circle and star were mounted on the four sides of the walls around the room as visual cues.

The Barnes maze testing consisted of a learning phase lasting four consecutive days and a probe trial. Four experimental groups of animals in experiment #1 performed the learning phase from post-injury days 14 to 17 and a probe trial on post-injury day 18. To be comparable, naïve animals performed the Barnes maze testing from 28 to 32 days after arriving at the facility, which was the same timing as other animals in Exp.1. During the learning phase (spatial reference memory), the rats were trained to learn the location of the target box for four consecutive days. The first day of the learning phase started with a habituation trial before the twice-daily learning trials. The rat was gently taken out from their home cage while the three bright lights were turned on. The rat was directly placed into the target box and stayed inside the target box for one minute. The rat was then removed from the target box to the center of the platform and allowed to explore the platform for about 10 seconds. Then, the rat was placed back to the escape box for 10 seconds and the bright lights were turned off. The rat was placed back in its home cage. The platform and all chambers were cleaned with 70% ethanol. The platform and all chambers were ensured to be dry when starting a new trial. The learning trials started five to 10 minutes following the habituation trial. The rat was taken from their home cage and put in the center of the maze under a "start chamber" which was a dark metal pot (23 cm in height x 24 cm in diameter) for 10 seconds. The bright lights were turned on during the holding period. After 10 seconds, the start chamber was lifted, and a learning trial began. The rat was allowed to freely explore the maze to locate the target box for five minutes. If the rat entered the escape box within five minutes, the rat was kept in the target box for one minute and the lights were turn off at the end of one minute. If the rat did not enter the target box within the five minutes, it was gently guided to the target box. The rat was then kept inside the target box for one minute. After spending one minute in the target box, the rats were then returned to the home cage. The maze was cleaned with 70% ethanol between each trial. After five to 10 minutes, the rat was taken out of the home cage and performed a second learning trial as describe in the first trial. The rats were trained to locate the target box with two trials per day for another three consecutive days. The location of the target box was fixed during all trials for all the rats. The SMART software (Panlab Harvard Apparatus) was used to track and measure the movement of the rats in the Barnes maze testing. In the learning phase, primary latency and primary distance were measured. Primary latency was defined as the latency in seconds to locate the target box. The distance travelled in centimeters to locate the target hole was termed primary distance. If the rat failed to locate the target chamber, a maximum latency of 300s (i.e., the total

duration of a trial) was used as primary latency and the total distance it travelled throughout the five minutes was used as primary distance.

Twenty-four hours after the last learning session, the rats were subjected to a probe trial (spatial memory recall), in which the target box was replaced with a regular chamber. The probe trial was conducted the same as a learning trial, except that the rats freely explored the maze for a total of five minutes. In the probe trial, in addition to the measurement of primary latency and primary distance, primary error was calculated. The number of none-target chambers the rat visited prior to locating the target box was named primary error. The platform was further divided into four quadrants: 1) target; 2) positive; 3) opposite; and 4) negative quadrants as shown in Figure 3.7.B. The target quadrant contained the target hole in the center while the other none-target quadrants contained all the other none-target holes. The percent time spent in target quadrant was calculated as following: [(time spent in the quadrant containing the target location) / overall duration of the probe trial] × 100%.

A) **B)**

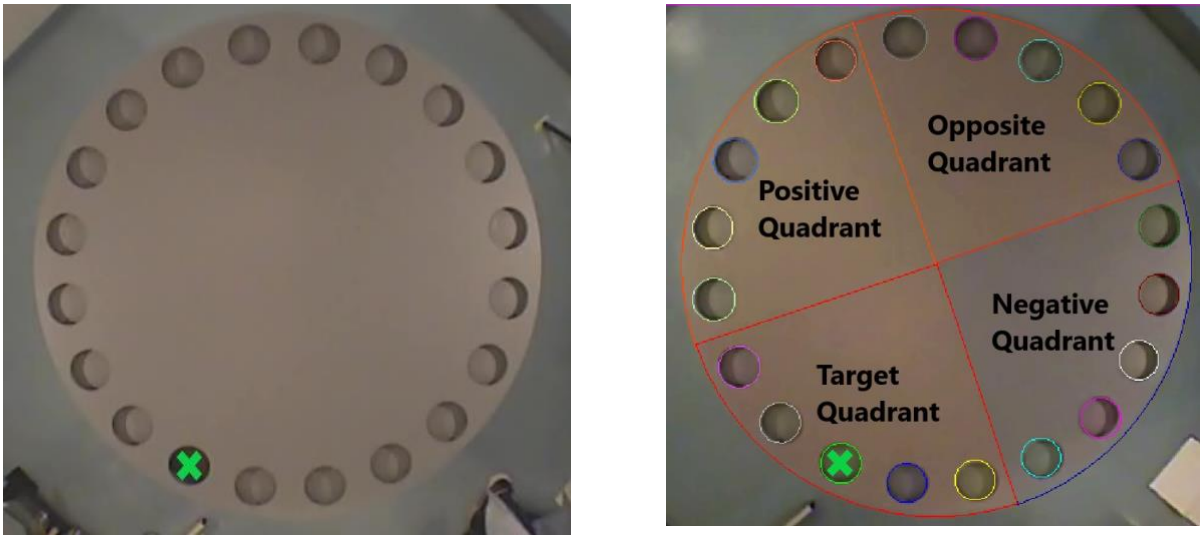

Figure 3.7 | The layout of Barnes Maze platform during A) learning phase and B) probe trial. During the learning phase, the rats were trained to learn the location of the target box for four consecutive days. The rats were allowed to freely explore the maze to locate the target box for a maximum of five minutes. Twenty-four hours after the last learning session, the rats were subjected to a probe trial, in which the target box was replaced with a regular chamber. The platform was further divided into four quadrants: 1) target; 2) positive; 3) opposite; and 4) negative quadrants. The rats freely explored the maze for a total of five minutes. Target escape box was indicated with "X".

3.7 Electrode Implantation

Rats underwent intracranial electrode implantation at an average of four and half months after FPI, ranging from three months seven days to nine months 27 days. The rat was anesthetized in a chamber with 5% isoflurane. The rat was then placed in a stereotaxic frame and continued to be anesthetized by isoflurane through mask. The appropriate level of anesthesia (1.5% - 3%) was achieved based on close monitoring with toe pinch, body color and breathing patterns. The head was shaved and cleaned before a midline incision was made. A Dremel hand drill was used to make small burr holes to implant electrodes (PlasticsOne). Two pairs of depth electrodes composed of 0.005 inch diameter tungsten wires were implanted within the craniotomy (coordinates: anterior posterior [AP] = -4.5; medial-lateral [ML] = -2.5; dorsal-ventral [DV] = 1.5) and CA1 of the hippocampus (coordinates: AP = -3.0; ML = 2.0; DV = 3.0). Screw electrodes were implanted in the ipsilateral frontal neocortex, ipsilateral parietal neocortex anterior to the craniotomy, and parietal neocortex posterior to the craniotomy. A singular depth electrode was implanted in contralateral parietal neocortex in the area homotopic to the craniotomy (AP = -4.5; ML = 2.5; DV = 1.5). Ground and reference electrodes (screw electrodes) were implanted over bilateral cerebellum. Figure 3.8 shows the configurations of the implanted electrodes.

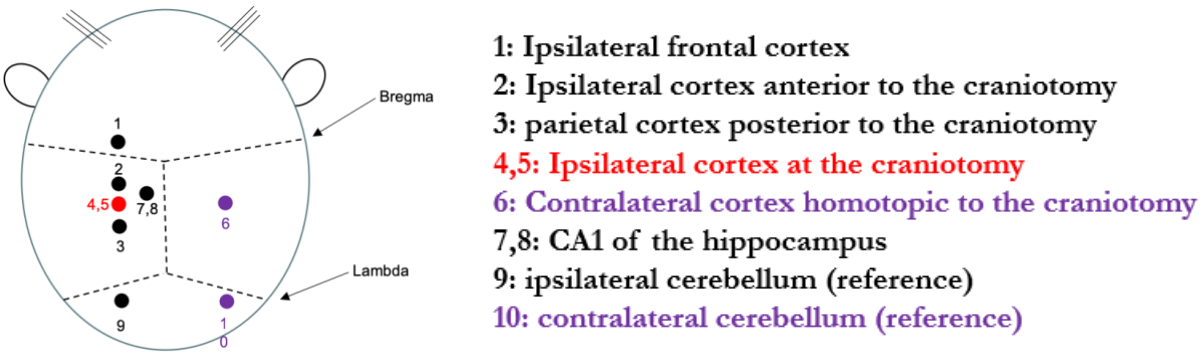

Figure 3.8 | Representation of implanted electrodes for PTZ seizure threshold test after FPI. Electrodes indicated in red were within the center of injured site, electrodes indicated in black were within in the hemisphere ipsilateral to the injury, and electrode indicated in purple were in the hemisphere contralateral to the injury.

The amphenol pins at the end of the electrodes were brought together and pinned into a headstage above the center of the skull. The headstage was secured with dental cement. Once the dental cement was dried, the rat was injected with meloxicam (1.0 mg/kg s.q.) and placed in a clean cage housing each individual rat. On post-implant day one, the rats were injected with a single dose of meloxicam (1.0 mg/kg s.q.) to relieve stress and pain related to the surgery. The rats were allowed to rest for at least a week prior to a monthly long-term video-EEG monitoring for the occurrence of spontaneous seizures. Since this long-term monitoring was outside of the scope of this thesis, these results are not included here. Some of the rats were subjected to seizure threshold testing following the long-term video-EEG monitoring as described below.

3.8 Seizure Susceptibility Testing

To determine seizure susceptibility after FPI, a subset of 32 rats in experiment #1 was subjected to PTZ seizure threshold testing approximately nine months following TBI (range: post-injury 263 to 312 days). Eight rats were selected from each experimental group that still had functioning electrodes to provide a good EEG signal. All PTZ seizure threshold tests were performed between 1 pm and 3 pm.

3.8.1 Preparation of Pentylenetetrazol

PTZ (1,5-pentamethylenetetrazole, Cat #: P6500-25G, Sigma-Aldrich) was dissolved in sterile 0.9% saline at a concentration of 12.5mg/ml. The PTZ solution was prepared no more than one day before the testing and stored at 4°C. On the testing day, the solution was taken out of the fridge and warmed up to the room-temperature before use.

3.8.2 Pentylenetetrazol Test Procedure

The test protocol was adapted from Kharatishvili and colleagues (2007). Prior to the injection, the rat was weighed to obtain an accurate body weight. The rat was then connected with a cable and injected with a single dose of PTZ (30mg/kg, i.p.). Past literature shows that PTZ doses ranging from 20 to 30 mg/kg are subconvulsant for adult male Sprague-Dawley rats (Golarai et al., 2001; Velisek, 2006). A more obvious difference regarding the frequency of convulsive seizures was observed between injured and control rats at a 30 mg/kg dose (Kharatishvili et al., 2007; Huusko et al., 2015).

Following the PTZ injection, each individual rat was placed into a modified cage (21 x 38 x 23 cm^3) where they could move freely. The cage was modified by cutting a hole in the lid, which allowed a cable to pass through and connect the rat with the EEG recording system (Acq*Knowledge* BIOPAC Systems, Inc., USA). The system was connected to MP160 amplifiers and filtered with either high-pass 1.0 Hz and low-pass 3.0K Hz cut offs, or high-pass 0.5 Hz and low-pass 100Hz. A video camera (LINKIT Security) was positioned above each cage and connected to a digital video recorder and a video monitor (Acer) to record the behavior of the animals. Video-EEG was recorded from implanted electrodes for 1 hour after the injection. An electrographic seizure was defined as a rhythmic discharge with high-amplitude, clear onset and offset, showing temporal evolution in wave morphology and amplitude, and lasting more than 5 seconds (Kharatishvili et al., 2007). During the 60 minutes immediately after PTZ injection, parameters including latency to the first seizure and total duration of seizure were calculated by manually examining the EEG patterns.

3.9 Cytokine Assay

Brain tissue from animals in experiment #2 was harvested to determine the concentrations of cytokines and chemokines after TBI. A total of 50 rats were randomly assigned to four experimental groups: 1) Sham; 2) TBI only; 3) TBI + LPS; 4) TBI + MINO. Rats from all experimental groups were sacrificed at either six hours after injury or post-injury day seven to investigate the TBI-related inflammation response in the subacute period.

3.9.1 Tissue Collection

Before harvesting the brain tissue, rats were perfused with ice-cold phosphate buffered saline (1x PBS, PH 7.4). Firstly, the rat was anesthetized with 5% isoflurane in a chamber, then moved to a flat surface facing dorsally and anesthetized with 5% isoflurane via the nosecone. The depth of sedation was closely monitored with toe pinch to ensure no painful senses during the procedure. The perfusion surgery was similar to Gage and colleagues (2012). Once the rat showed no response to the toe pinch, a lateral incision through the abdominal area right below the xiphoid was made to expose the heart. A 16-gauge needle was inserted into the apex of the left ventricle and the right atrium was then cut. The end of the needle was connected with an outflow tube of a perfusion pump (MASTERFLEX® L/S Economy Drive, Cole-Palmer

Instrument Co.). The rat was transcardially perfused with approximately 250 -300 ml of 1xPBS until the liver was light brown. Once the perfusion was complete, the head was decapitated. After removing the skull, the whole brain was kept wet with the ice-cold 1xPBS on a petri-dish above a bucket of ice. The following tissues were dissected out: 1) neocortical tissues on the injury site (ipsilateral neocortex) and 2) ipsilateral hippocampus. Each tissue sample was immediately stored in a 1.5 ml microcentrifuge tube (Fisherbrand) and flash-frozen in liquid nitrogen. The tissues were stored in -80$_{o}$C until determination of total protein and cytokines.

3.9.2 Preparation of Homogenates

The brain tissue was homogenized in a cell lysis buffer compatible with the multiplex assay. The recipe of the lysis buffer was adapted from a paper by Fox and colleagues (2005) and consisted of 20mmol/L Tris-HCL (Sigma Aldrich, pH 7.5), 150 mmol/L NaCl (Sodium Chloride, BioShop), 1 mmol/L PMSF (BioShop), 0.05% Tween-20 (Sigma Aldrich), and a cocktail of protease inhibitors (Roche). A 10x stock solution was made in advance. Furthermore, a PMSF stock solution (100 mmol/L) was made by dissolving PMSF powder in 100% ethanol (Caledon). Fresh 1x cell lysis buffer was diluted from the 10x stock solution and stock PMSF solution with the addition of protease inhibitor cocktail tablets (Roche).

Frozen tissue samples were defrosted on ice. The weight of each brain sample was calculated by weighing the microcentrifuge tube prior to and after receiving the tissues. For every 100 mg of tissue, approximately 600 µl cell lysis buffer was added into the tube. Then, a hand-held homogenizer (Cat #: 47747-370, VWR®Pellet Mixer) was used to homogenize the tissue while on ice. A clean pestle (Argos Technologies, Inc.) was used for each brain sample. Once there was no sign of tissue clumps, tubes were agitated on a planar shaker (Thermo Scientific MaxQ$_{TM}$ 2000) on LOW setting for 30 minutes at 4°C. The tube was centrifuged (Eppendorf Centrifuge 5417R)) at 13,000 rpm for 10 minutes at 4°C. Debris was removed by transferring the supernatant to a new microcentrifuge tube. The supernatant was the desired tissue lysates from which the aqueous extract was made and stored at -80°C until protein assay and multiplex assay.

3.9.3 Quantification of Total Protein Concentration

3.9.3.1 Principle

The concentration of protein of each sample was determined using the *DC* (detergent compatible) Protein Assay Kit (Bio-Rad). This protein assay is a colorimetric assay for measuring total protein concentrations specifically for samples solubilized by detergent (Bio-Rad Instruction Manual, *DC* Protein Assay). The principle is based on the well-known Lowry assay (Lowry et al., 1951), with several improvements including faster color development and maintaining color changes for longer time. As documented in the Lowry assay (Lowry et al., 1951), color development results from two steps. Firstly, protein reacts with copper in an alkaline medium. Subsequently, the copper-treated protein reduces Folin reagent, which leads to several reduced species characterized with a blue color with an absorbance ranging from 405 to 750 nm (Lowry et al., 1951; Peterson, 1979).

3.9.3.2 Protein Quantification Procedure

The manufacturer's instructions were followed to perform the microplate protein assay. All the reagents of the kit were warmed to room temperature (RT, 20 - 25°C) before being used in the assay. The aqueous extract of tissue lysates was taken out of the -80°C freezer and defrosted on ice. The assay was run in duplicate in a clean and dry 96-well microplate. The placement of standards and samples in the well was determined and written down in a microplate diagram. Lyophilized bovine gamma globulin (BGG, Bio-Rad Protein Assay Standard) was rehydrated to prepare stock protein solutions at a concentration of 1.56 mg/ml and the stock solutions were stored at -20°C. Dilutions of BGG standard were prepared in the same cell lysis buffer used for homogenizing the brain tissues. A standard curve was prepared for every assay, which included the following known concentrations of the BGG standard solutions: 1) 0.195 mg/ml; 2) 0.39 mg/ml; 3) 0.78 mg/ml; 4) 1.17mg/ml; 5) 1.56 mg/ml (i.e., stock protein solution). A 0 mg/ml of BGG standard was used as a blank, by adding the cell lysis buffer to the well. A working reagent A' was prepared by adding 20 µl of reagent S (i.e., a surfactant solution) to each ml of reagent A (i.e., an alkaline copper tartrate solution) that would be enough for running the assay, which was 25 µl of reagent A' per sample. The working reagent A' was vortexed well to ensure an absence of undissolved precipitate before pipetting.

Firstly, 5 µl of standards and samples were pipetted into the microplate. Following the addition of 25 µl of working reagent A' into each well, 200 µl of reagent B (i.e., a dilute Folin reagent) were pipetted into each well. The microplate was incubated in the dark for 15 minutes before the reading. At the end of the incubation, absorbances of the plate were measured by the SPECTROstar*Nano* microplate reader (BMG LABTECH) with the wavelength set to 750 nm. The MARS software (BMG LABTECH) was used to correct the raw absorbance of each same based on the absorbance of the blank and a linear regression was plotted using the corrected absorbances of known standard concentrations. The protein concentration in the unit of mg/ml was interpolated for each sample based on the plotted linear regression. For each sample, a mean protein concentration was calculated by averaging the duplicates.

3.9.4 Determination of Cytokines and Chemokines

The MILLIPLEX®MAP Rat Cytokine/Chemokine Magnetic Bead Kit (RECYTMAG-65K, EMD Millipore) was used to quantify the cytokines and chemokines early after TBI. Compared to the traditional enzyme-linked immunosorbent assay (ELISA), the multiplex assay simultaneously measures the concentrations of multiple cytokines and chemokines in the same sample. Thus, the multiplex assay has advantages, since it requires lower sample volumes and has higher efficiency regarding time and labor.

3.9.4.1 Multiplex Assay Design

The principle of this multiplex assay is based on the Luminex® multi-analyte profiling (xMAP®) technology, which is a bead-based multiplex assay (Luminex, 2017). Magnetic bead microspheres (MagPlex®) are color coded with multiplex fluorescent dyes, leading to the creation of distinct colored bead sets. Each of the bead sets is coated with a specific capture antibody and consists of 80 6.45 µm magnetic polystyrene microspheres. After the beads capture target analytes in the samples, biotinylated detection antibodies are added to create specific bindings to the analyte-capture antibody complex on the bead sets. To complete the reaction on the surface of each bead, a reporter molecule, Streptavidin-Phycoerythrin conjugate is introduced to incubate with the "sandwich" complex (i.e. analyte-capture antibody-biotinylated detection antibody complex).

To obtain and analyze the data, the Luminex®200™, a flow-cytometry based instrument, was used. Two lasers in the instrument excite and detect different components of the complex, while the beads in each well pass through the Luminex® sheath fluid (EMD Millipore). A red laser with a wavelength of 635 nm is used to identify each bead region based on its internal dye concentrations. A green laser with a wavelength of 525 nm is used to quantify the signal of the reporter molecule to determine whether the binding event occurs or not. The concentration of interested analytes in each sample can be determined by identifying each specific bead and the amount of fluorescence signal associated with each bead.

3.9.4.2 Immunoassay Procedure

The multiplex assay was performed following the instructions in the manual. The assay was run in duplicate as recommended by the manufacture. The placement of each well including standards, controls and samples was written down in a microplate diagram in advance. All reagents of the multiplex kit were allowed to warm to RT before use. There are six analytes of interest in the current study as follows:1) IL-1β; 2) TNFα; 3) IL-10; 4) MCP-1/CCL2; 5) MIP-1α/CCL3; 6) RANTES/CCL5.

Preparation of several reagents was required before running the assay. Antibody-immobilized beads were included in the kit either as premixed beads or individual vials of beads. If beads were premixed by the manufacture, the premixed bead bottle was sonicated for 30 seconds followed by one minute of vortex. In the case of individual vials of beads, each bead vial was sonicated for 30 seconds and then vortexed for one minute. The beads were mixed by adding 60 µl from each of six bead vials and 2.64 ml Bead Diluent to the Mixing Bottle, resulting a final volume of 3.0 ml. The Mixing Bottle was vortexed well after adding all reagents. Two Quality Controls (QCs) were included in the kit to help qualify the performance of each assay. Each QC vial was reconstituted with 250 µl deionized water, followed by multiple shaking and vortex. The reconstituted QC vials were allowed to sit for at least five to 10 minutes before being used. Wash Buffer, which was used to wash out unbound antibodies, was prepared by diluting 60 ml of 10X Wash Buffer with 540 ml deionized water. After dilution, the Wash Buffer was mixed well.

Preparation of Standards started with reconstituting the Rat Cytokine/Chemokine Standard with 250 ul of deionized water. The reconstituted vial was inverted several times and vortexed for 10

seconds. This vial was used as Standard 7. Six 1.5 ml microcentrifuge tubes were labelled, and 120 ul of Assay buffer was added to each tube. Then, 40 ul of Standard 7 was added to the Standard 6 tube and mixed well before transferring 40 ul of Standard 6 to the Standard 5 tube. The Standard 5 tube was mixed well and transferred 40 ul to Standard 4 tube. The preparation of Standard 3, 2 and 1 was the same by transferring 40 ul of previous Standard and mixed well before transferring to the next lower concentration. All prepared dilutions were used within one hour after preparation. A summary of preparation of Standards is shown in Table 3.3 and the concentration of each interested cytokine/chemokine for each standard is indicated in Table 3.4.

Table 3.3 | The preparation of a serial dilutions of standard in the multiplex assay.

Standard Number	Amount of Deionized Water or Assay Buffer To Add	Amount of Standard to Add
Standard 7	250 µl of Deionized Water	0 µl
Standard 6	120 µl of Assay Buffer	40 µl of Standard 7
Standard 5	120 µl of Assay Buffer	40 µl of Standard 6
Standard 4	120 µl of Assay Buffer	40 µl of Standard 5
Standard 3	120 µl of Assay Buffer	40 µl of Standard 4
Standard 2	120 µl of Assay Buffer	40 µl of Standard 3
Standard 1	120 µl of Assay Buffer	40 µl of Standard 2

Table 3.4 | The concentration of analyte in each standard.

Standard Number	MIP-1α, IL-1β, TNFα (pg/ml)	RANTES (pg/ml)	IL-10 (pg/ml)	MCP-1 (pg/ml)
Standard 1	2.4	4.9	7.3	29.3
Standard 2	9.8	19.5	29.3	117.2
Standard 3	39.1	78.1	117.2	468.8
Standard 4	156.3	312.5	468.0	1,875.0
Standard 5	625.0	1,250.0	1875.0	7,500.0
Standard 6	2500.0	5,000.0	7500.0	30,000.0
Standard 7	10,000.0	20,000.0	30000.0	120,000.0

After completing the preparation of all needed reagents and Standards, 200 µl of Assay Buffer were added into each well. The plate was sealed and mixed on a plate shaker (VWR® Microplate Shaker) at a speed of 750 rpm at RT for 10 minutes. The plate was then inverted and tapped gently onto clean paper towels to remove Assay Buffer from all wells. Standards or QCs

(25 µl each) were added to their corresponding wells. Assay Buffer was used as the background at a concentration of 0 ng/ml. Subsequently, 25 µl of Assay Buffer were pipetted into each sample well, followed by adding 25 µl of the same cell lysis buffer to the background, standards, and control wells. The sample (25µl) was added to each appropriate well. The Mixing Bottle was vortexed well and 25 µl of the Premixed or Mixed Beads were added to each well. The plate was sealed and wrapped with foil and agitated on a plate shaker at the speed of 750 rpm at RT for two hours.

After the first incubation, the plate was washed twice using a handheld magnet (EMD Millipore). To wash the plate, it was first rested on magnet for 60 seconds, allowing the magnetic beads to settle down to the bottom of the well. The contents were removed by inverting the plate and gently tapping on clean paper towels to remove remaining liquid. The plate was removed from magnet and each well was filled with 200 µl of Wash Buffer. The plate was then manually shaking for one minute and reattached to magnet allowing for 60 seconds settling. The contents were removed as descried previously.

Following the wash steps, 25 µl of Detection Antibodies were added to each well and the plate was sealed as well as covered with foil. The second incubation occurred by agitating the plate on the plate shaker (750 rpm) for one hour at RT. There were no wash steps following the second incubation. Instead, 25 µl of Streptavidin-Phycoerythrin were added to each well and the plate was sealed and covered with foil. The third incubation was on the plate shaker (750 rpm) for 30 minutes at RT. The plate was washed twice following the same steps described above after the third incubation. Wash Buffer (125 µl) was added to all wells and the beads were resuspended by shaking on the shaker (750 rpm) for five minutes at RT. Finally, the plate was run on Luminex®200™ with xPONENT software. The Median Fluorescent Intensity (MFI) data was analyzed using a five-parameter logistic to calculate the concentrations of analytes in samples in a unit of pg/ml. A mean concentration was obtained by averaging the duplicate. Each sample was normalized by its total protein amount, having the final results expressed as pg of interested cytokines or chemokines per mg of total protein.

3.10 Statistical Analysis

All data were analyzed using GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, California USA). The Shapiro-Wilk test was used to test the normality of the data and Brown-Forsythe test was used to test the assumption of equal group variance. The non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test was used in the analysis of 1) righting reflex; 2) composite neuroscore; 3) rotarod test; 4) Barnes maze test; and 5) PTZ-induced seizure threshold test. To compare multiple time points within an animal group, Freidman test followed by Dunn's multiple comparisons test was performed. Novel object recognition data passed the normality test. Thus, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed on these data. The concentrations of cytokines and chemokines at each time point were analyzed by one-way ANOVA followed by Tukey's test.

3.10.1 Data Presentation

Since non-parametric methods compare the sum of ranks between groups (Motulsky, GraphPad Statistics Guide), data analyzed by Kruskal-Wallis test were represented as median ± [95%CI]. Parametric methods compare means between groups (Motulsky, GraphPad Statistics Guide). Data analyzed by ANOVA were displayed as mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$ for all tests.

4 Results

4.1 Weights and Injury parameters

Figure 4.1 illustrates the experimental design and indicates the number of rats in each experimental arm of the study. We used a total of 162 rats and had an immediate mortality of 23%. The mean weight of the animals at the time of injury was 379g, ranging from 309g to 441g. The injury angle was between 13.0 to 14.5 degrees, resulting in a force of the fluid pressure between 2.0 atm to 2.5 atm. The duration of apnea was 0 to 130 second. Among the rats that underwent both behavioral testing and PTZ-induced seizure susceptibility testing, a total of 6 died spontaneously, or were sacrificed due to morbidity (from > 24 hours post-injury to PTZ seizure threshold testing) of the experiment. These six mortalities included 3 TBI + MINO rats that died 49, 53 and 121 days following the injury, one TBI-only rat sacrificed on post-injury day 158 due to sickness following electrode implantation, one TBI + MINO rat sacrificed on post-injury day 59 due to bloated abdominal cavity and one TBI-only rat sacrificed on post-injury day 210 due to difficulty in breathing.

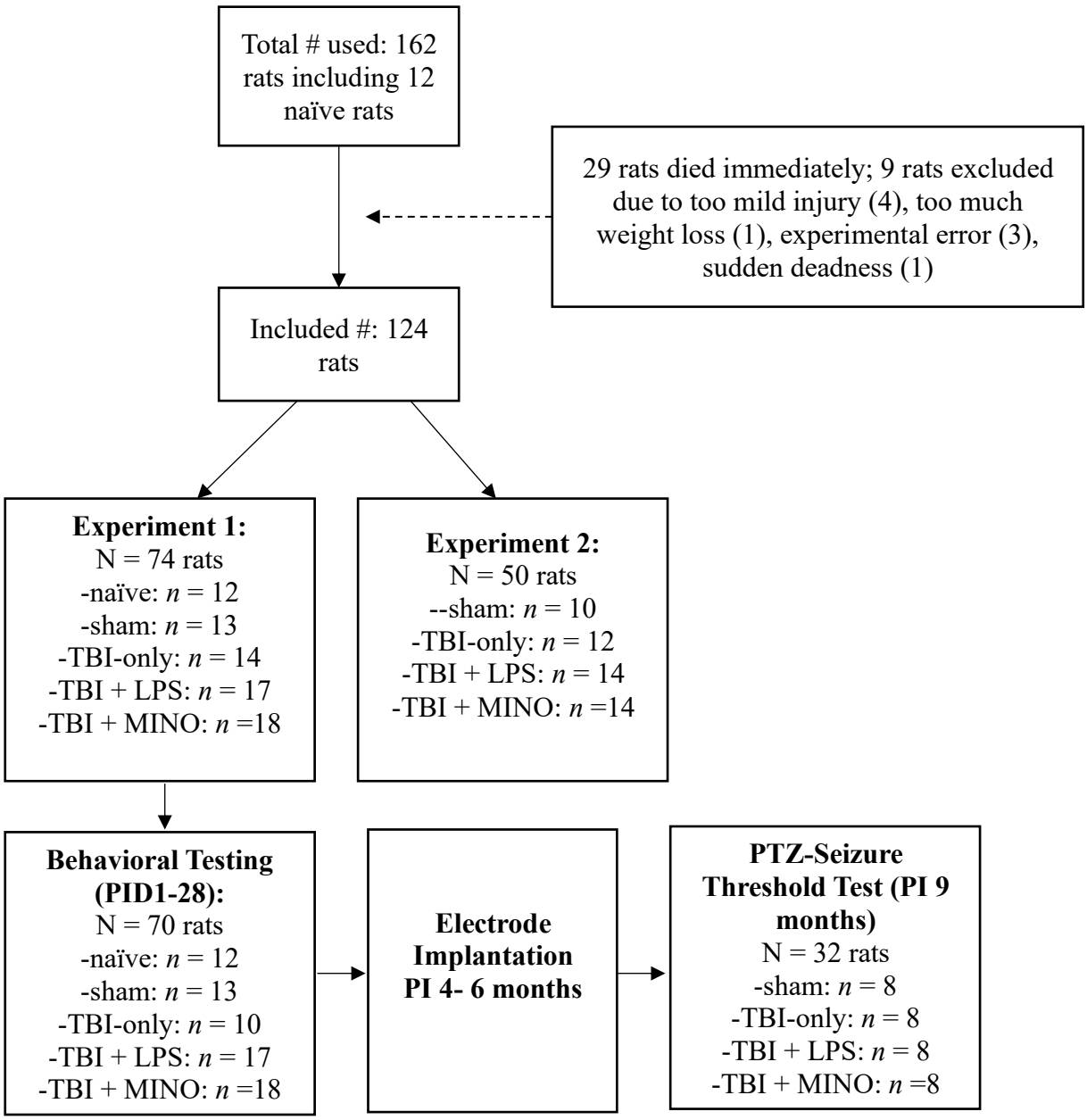

Figure 4.1 | Experimental design indicating the number of rats in each group per experiment. Abbreviation: LPS (liposaccharides), MINO (minocycline), PI (post-injury), PID (post-injury day), PTZ (pentylenetetrazol).

4.2 LPS did not alter initial severity of injury

The initial injury severity was assessed by measuring righting reflex immediately after the induction of FPI in all injured animals. Rats in the TBI + LPS group received the LPS injection before assessment of the righting reflex. In the TBI + MINO group, the duration of regaining the righting reflex was measured before the injection of minocycline. Therefore, only LPS administration, but not minocycline treatment, was involved in this measurement.

We found no difference in the righting reflex time between any of the injured groups immediately after injury (Figure 4.2). This indicates the administration of LPS prior to injury did not alter initial injury severity. The median latency to demonstrate a righting reflex (± 95% CI) for each injured group was as following: 1) TBI-only: 1113s ± [665,1536]; 2) TBI + LPS: 1100s ± [829,1620]; and 3) TBI + MINO: 1061s ± [896,1388].

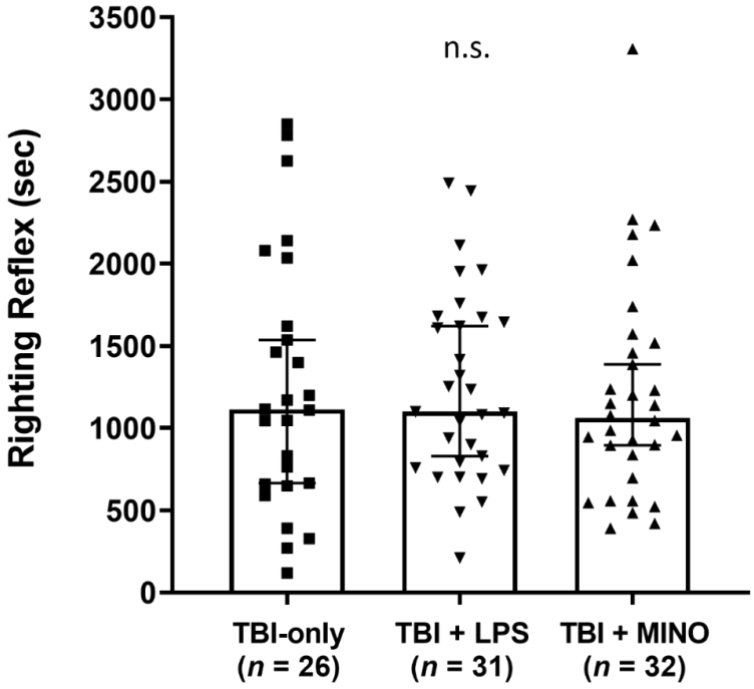

Figure 4.2 | Evaluation of initial injury severity using righting reflex. LPS did not alter initial injury severity. There was no difference in the righting reflex between any of the injured groups, demonstrating no difference in the injury severity immediately after FPI. Data were reported as median ± 95% CI with individual data points for each injured group. Abbreviation: n.s. indicated $p > 0.05$ in Kruskal-Wallis test.

4.3 Both LPS and MINO increased concentrations of IL-1ß, CCL2, CCL3 and CCL5 six hours after FPI compared to sham animals, while MINO lowered hippocampal concentrations of CCL3 and CCL5 compared to injured-only rats one week after FPI

The expression of various cytokines and chemokines in both the ipsilateral cortex and hippocampus was evaluated to investigate the inflammatory response after the induction of TBI in each experimental group at post-injury six hours and day seven. Data that were under Minimum Detectable Concentration (MinDC) were not included in the analysis. More samples from post-injury day seven did not reach MinDC than from post-injury day six. Figure 4.3 A-F shows the concentration of each cytokine in both the cortex and hippocamps at six hours following injury. The concentration of each cytokine in the both cortex and hippocampus on post-injury day seven is shown in Figure 4.4 A-F.

We observed acutely elevated expression of several cytokines and chemokines post-TBI. The concentrations of IL-1ß, MCP-1/CCL2, MIP-1α/CCL3, and RANTES/CCL5 were increased six hours after FPI. We did not observe significant group differences in TNF-α and IL-10 levels. IL-1ß levels were acutely elevated in both the cortex and hippocampus following injury compared to the sham group. MINO attenuated the increase of IL-1ß at this time point in the cortex but not the hippocampus. Specifically, six hours after FPI, the mean concentration of IL-1ß was 70.4 ± SEM 9.8 pg/mg protein in the sham cortex and was significantly higher at 224.8 ± 47.0 pg/mg in the TBI-only cortex ($p < 0.05$), and 299.1 ± 30.2 pg/mg in the TBI + LPS cortex ($p < 0.001$). In the hippocampus, the mean concentration of IL-1ß was 83.6 ± 4.1 pg/mg in the sham group versus 376.0 ± 61.0 pg/mg in the TBI-only group ($p < 0.05$), 414.5 ± 66.0 pg/mg in the TBI + LPS group ($p < 0.05$), and 357.3 ± 64.4 pg/mg in the TBI + MINO group ($p < 0.05$).

A higher level of MIP-1α/CCL3 expression was observed in both the cortex and hippocampus following injury compared to the sham group. In the cortex this increase was not influenced by either LPS or MINO. We did not find increased CCL3 levels in the TBI-only hippocampus compared to the sham group. However, both LPS and MINO groups had higher CCL3 levels in the injured hippocampus compared with the sham group. Specifically, six hours after FPI, the mean concentration of CCL3 was 15.3 ± 1.2 pg/mg protein in the sham cortex and was

significantly higher at 194.6 pg/mg ± 28.5 pg/mg in the TBI-only cortex ($p < 0.05$), 221.3 ± 19.1 pg/mg in the TBI + LPS cortex ($p < 0.01$) and 193.3 ± 61.0 pg/mg in the TBI +MINO cortex ($p<0.05$). In the hippocampus, the mean concentration of CCL3 was 3.1 ± 1.0 pg/mg in the sham group versus 258.3 ± 26.4 pg/mg in the TBI+LPS group ($p < 0.01$), and 249.3 ± 62.1 pg/mg in the TBI + MINO group ($p <0.01$).

We also observed acutely elevated concentrations of MCP-1/CCL2 and RANTES/CCL5 following injury for both the TBI + LPS and TBI + MINO groups compared to the sham group. The higher concentration was not observed in the TBI-only group compared to the sham group. Higher levels of CCL2 expression in both the cortex and hippocampus were observed, while an increased concentration of CCL5 was only observed in hippocampus. Specifically, six hours after FPI, the mean concentration of CCL2 was 53.4 ± 6.2 pg/mg in the sham cortex and was significantly higher at 474.4 ± 60.3 pg/mg in the TBI + LPS cortex ($p < 0.01$), and 371.9 ± 68.6 pg/mg in the TBI + MINO cortex ($p < 0.05$).In the hippocampus, the mean concentration of CCL2 was 61.4 ± 35.5 pg/mg in the sham group versus 705.7 ± 66.3 pg/mg in the TBI + LPS group ($p < 0.05$), and 830.5 ± 182.5 pg/mg in the TBI +MINO group ($p < 0.01$). The mean concentration of CCL5 was 6.6 ± 0.6 pg/mg in the sham hippocampus and was significantly higher at 12.1 ± 1.8 pg/mg in the TBI + LPS hippocampus ($p < 0.05$), and 10.8 ± 1.3 pg/mg in the TBI + MINO hippocampus ($p < 0.05$)

Seven days after FPI, the concentrations of all cytokines and chemokines in the cortex returned to a level where no difference between groups was observed anymore. We observed that MINO appeared to modulate levels of certain chemokines expression, CCL3 and CCL5 in the hippocampus. Compared to the TBI-only rats, the TBI + MINO group had significantly lower levels of CCL3 and CCL5 expression in the injured hippocampus. Specifically, seven days after injury, the mean concentration of CCL3 was 18.5 ± 4.1 pg/mg in the TBI-only hippocampus and was significantly lower at 7.1 ± 1.1 pg/mg in the TBI + MINO hippocampus ($p <0.05$). The mean concentration of CCL5 was 9.9 ± 0.9 pg/mg in the TBI-only hippocampus and was significantly lower at 5.2 ± 1.2 pg/mg in the TBI + MINO hippocampus ($p < 0.05$). The concentration of each cytokine in both the cortex and hippocampus on post-injury day seven was shown in Figure 4.4 A-F.

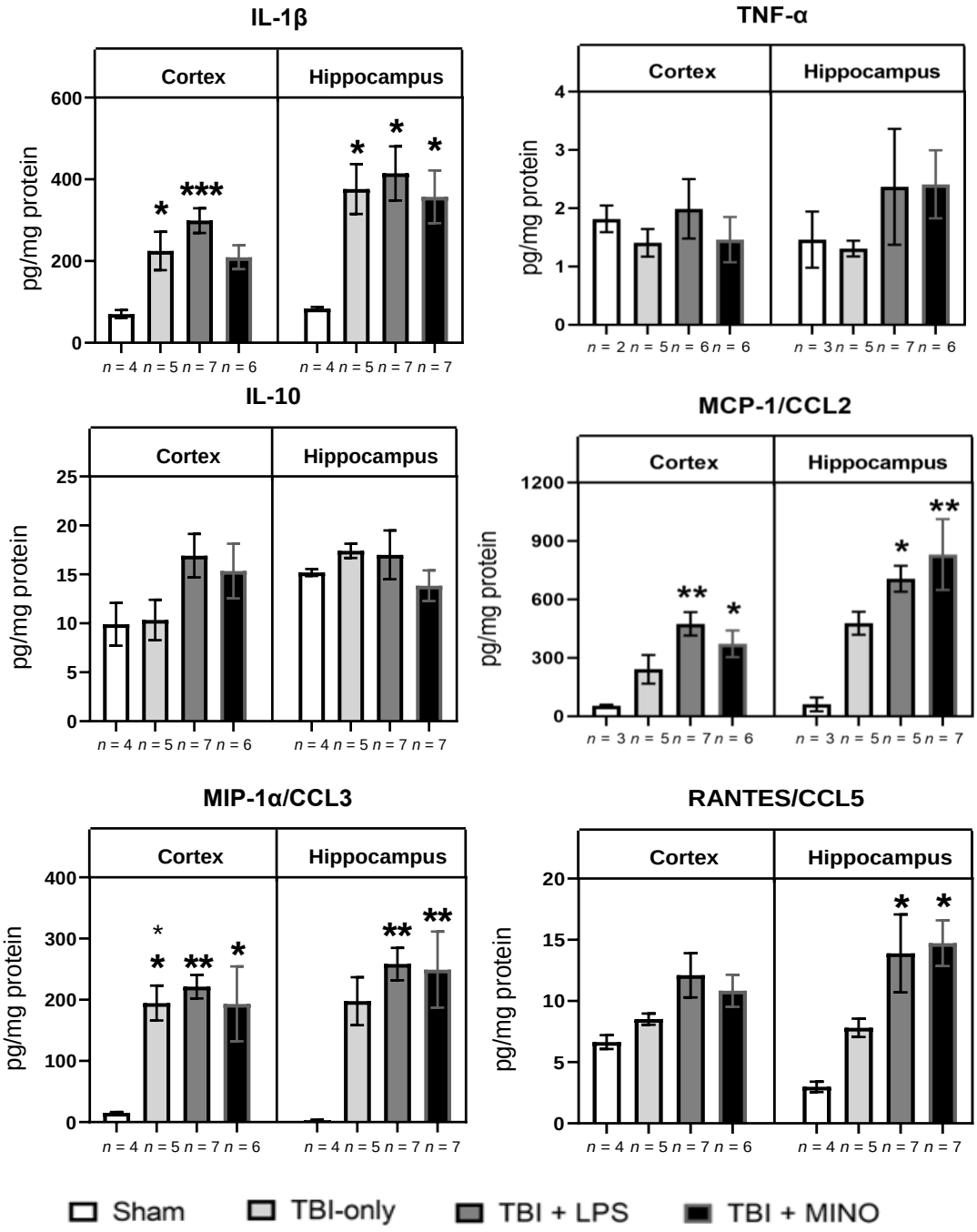

Figure 4.3 | The concentration of A) IL-1ß, B) TNF-α, C) IL-10, D) MCP-1/CCL2, E) MIP-1 α/CCL3, and F) RANTES/CCL5 in both the cortex and hippocampus for each experimental group at six hours following TBI. Increased concentrations of IL-1ß and MIP-1α/CCL3 were observed in all injured groups compared to the sham group. The concentrations of MCP-1/CCL2 and RANTES/CCL5 were higher in the TBI + LPS and the TBI + MINO groups compared to sham. No significant group difference was observed for TNF-α and IL-10 at this time point. Data are reported as mean ± SEM. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to the sham group, One-way ANOVA followed by Tukey's multiple comparisons test.

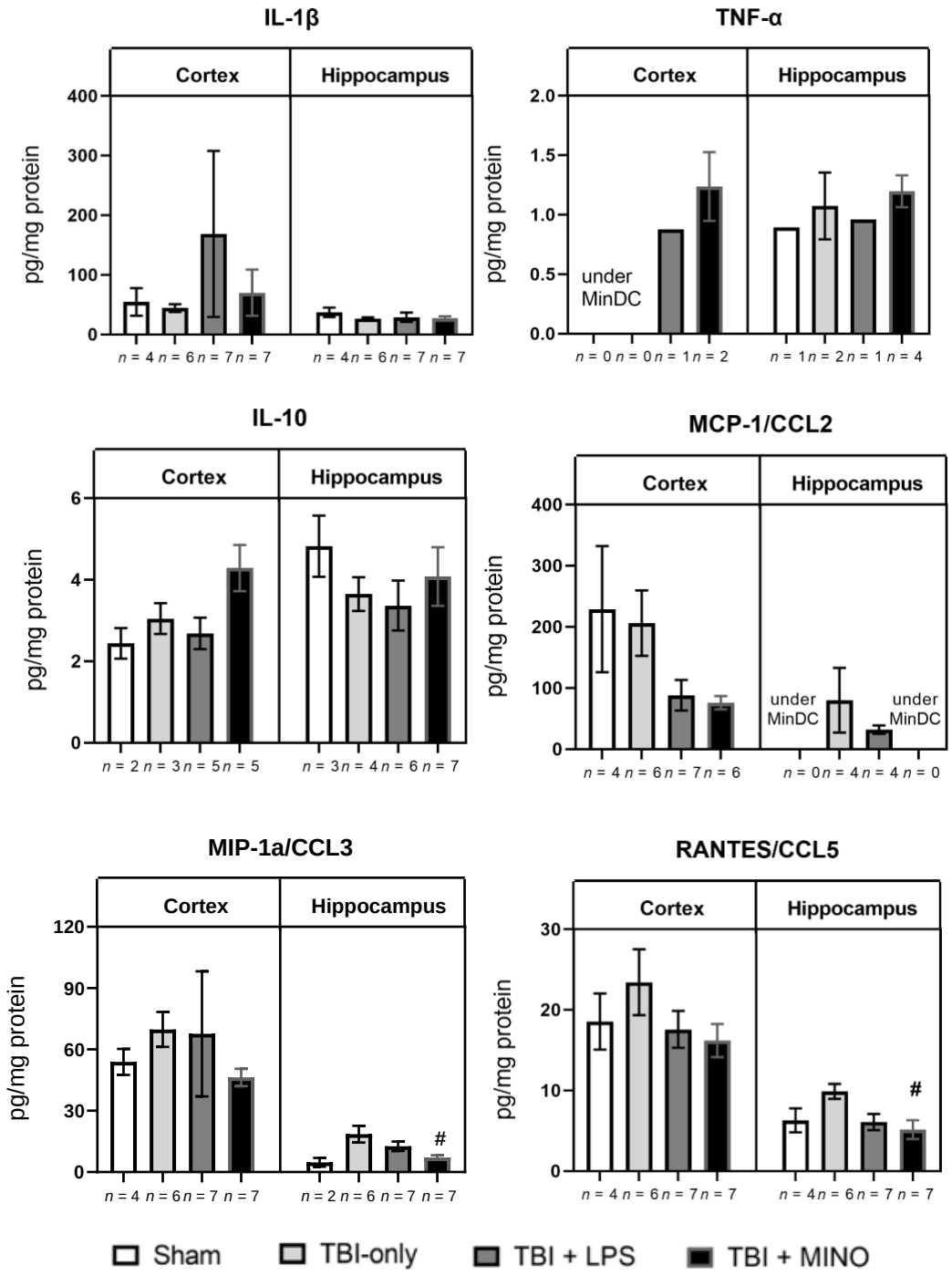

Figure 4.4 | The concentration of A) IL-1ß, B) TNF-α, C) IL-10, D) MCP-1/CCL2, E) MIP-1 α/CCL3, and F) RANTES/CCL5 in both cortex and hippocampus for each experimental group at post-injury day seven. No group difference in the cortex was observed seven days after TBI. The TBI+MINO group had significantly decreased concentrations of CCL3 and CCL5 in hippocampus compared to the TBI-only rats. Data are reported as mean ± SEM. Statistical significance: #$p < 0.05$, compared to the TBI-only group, One-way ANOVA followed by Tukey's multiple comparisons test. Abbreviation: Minimum Detectable Concentration (MinDC).

4.4 LPS slowed down neurological motor recovery four weeks following TBI

Neurological motor function was evaluated by using composite neuroscore. We excluded four sham animals from analysis because a different mat surface of the angle board was used. Composite neuroscores for all experimental groups prior to injury (baseline) and on post-injury day two, eight,15 and 28 are shown in Figure 4.5. We did not observe differences in baseline scores between any groups. All injured groups showed deficits in neurological motor function as compared to sham animals at the early time points, post-injury day two and day eight. This was not altered by either LPS or MINO treatment. Both the TBI + LPS and TBI + MINO groups continued to show impairment in motor performance compared to the sham group at later time points, post-injury day 15 and day 28. LPS led to persistent neurological motor dysfunction four weeks post-TBI not seen in other injured groups. Specifically, the TBI+LPS group had lower neuroscores compared to the sham group at all-injury testing days (Kruskal-Wallis: $p < 0.0001$ for post-injury day two, eight, and 28; $p < 0.01$ for day 15). Dunn's multiple comparisons test revealed that all injured groups had a significantly lower composite neuroscore than sham animals on post-injury day two ($p < 0.01$, 0.0001, 0.01 for sham versus TBI-only, TBI + LPS and TBI + MINO, respectively) and day eight ($p < 0.05$, 0.0001, 0.01 for sham versus TBI-only, TBI +LPS and TBI + MINO, respectively). While the TBI-only group showed no difference from the sham group on post-injury day 15 or 28, both the TBI + LPS and TBI + MINO groups continued to perform worse than sham animals at both time points (D15: $p < 0.001$ and 0.05, respectively; D28: $p < 0.00$ and 0.01, respectively). The TBI + LPS group was the only injured group that did not recover to the same level as the TBI-only group on post-injury day 28 (median composite neuroscores 22.7 ± 95% CI [20.0,25.0] versus 26.0 ± [25.0,27.7], $p < 0.05$).

We also observed all injured groups had neurological motor impairment compared to their baseline at early time points, post-injury day two and eight. This was not influenced by LPS or MINO. Both the TBI + LPS and TBI +MINO groups continued to show impairment in motor performance than their baseline on post-injury day 15. All injured groups showed neurological motor recovery starting on post-injury day 15 and continued their recovery four weeks following TBI. We observed sham animals had stable neurological motor function across time points. Specifically, all injured groups: the TBI-only, TBI + LPS, and TBI + MINO groups showed significant differences between time points (Friedman test: $p <0.0001$ for all 3 groups).

Dunn's multiple comparisons test showed that on post-injury day two and eight, all injured groups had a significantly worse neuroscore than their baseline. There was no difference observed between baseline and the last two time points (i.e., post-injury day 15 and 28) for TBI-only group. Both the TBI + LPS and TBI + MINO groups continued to perform worse than their baseline on post-injury day 15 (TBI + LPS: 28.0 ± [27.0,28.0] at baseline versus 21.0 ± [17.7, 24.0] on post-injury day 15, $p<0.05$; TBI + MINO: 28.0 ± [28.0,28.0] at baseline versus 25.0 ± [20.3, 26.0] on post-injury day 15, $p < 0.05$). All injured groups started to show recovery on post-injury day 15 and continued to recover on day 28 when comparing to their performance on day two (Post-injury day 15 versus day two: $p < 0.05$, 0.001 and 0.001 for the TBI-only, TBI + LPS, and TBI + MINO groups, respectively; Post-injury day 28 versus day two: $p < 0.0001$, 0.0001, and 0.001 for the TBI-only, TBI + LPS, and TBI + MINO groups, respectively).

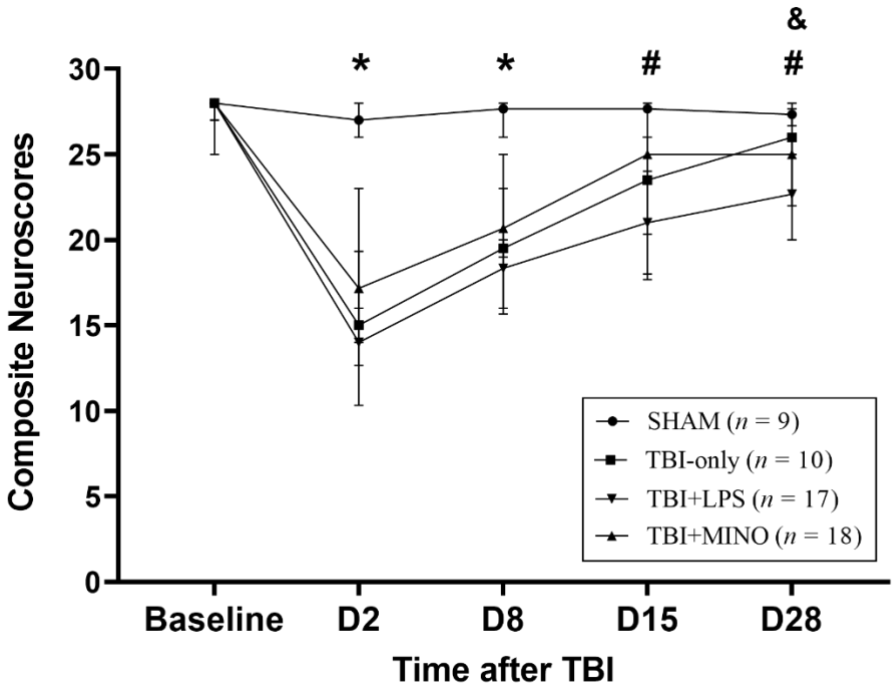

Figure 4.5 | Evaluation of neurological motor function and recovery using composite neuroscores across various post-injury days. All injured groups showed similar impairment in neurological motor function relative to the sham group on post-injury day two and eight. Both the TBI + LPS and TBI + MINO groups, but not the TBI-only group, continued to perform worse than sham animals on post-injury day 15 and 28. The TBI + LPS group had not recovered to the same level as the TBI-only group on post-injury day 28. Data are reported as median ± 95% CI. Statistical significance: $*p < 0.05$, all injured groups versus the sham group; $\#p<0.05$, the TBI + LPS or TBI + MINO groups versus sham animals; $\&p < 0.05$, the TBI + LPS group versus the TBI-only group (Kruskal-Wallis followed by Dunn's multiple comparisons test).

4.5 Both LPS and MINO ameliorated sensorimotor impairment one day following TBI

Sensorimotor coordination and balance were evaluated by the rotarod test. Figure 4.6 displays the latency on the rotating rod until the animal fell off at baseline (prior to injury) and on post-injury day one, seven, and 14 for the four experimental groups. We did not observe any group differences at baseline. We found impairment in sensorimotor coordination and balance one day following FPI, which was not seen in the TBI + LPS and TBI + MINO groups. Specifically, the TBI-only group (median latency 1.8s ± 95% CI [0.7, 24.7]) had a significantly shorter latency on the rotating rod than sham animals (22.3s ± [8.0, 34.7]**)** on post-injury day one ($p < 0.05$). No impairment in sensorimotor function in any group one or two weeks following TBI was found.

When comparing within each group, all injured groups performed worse than their baseline one day following FPI, regardless of treatment. We observed sensorimotor recovery in the TBI + LPS and TBI + MINO groups two weeks after injury. Specifically, all injured groups had a significantly shorter latency on the rotating rod on post-injury day one as compared to their baseline (the TBI-only group: 41.7s ± [29.7, 66.0] at baseline versus 1.8s ± [0.7, 24.7] on post-injury day one, $p < 0.0001$; the TBI + LPS group: 31.0s ± [23.7, 55.0] at baseline versus 13.3s ± [3.7, 23.0] on post-injury day one, $p < 0.0001$; the TBI + MINO group: 41.2s ± [27.3, 59.7] at baseline versus 8.8s ± [2.7, 14.7] on post-injury day one, $p < 0.0001$). On post-injury day seven, both the sham and TBI-only groups performed worse than their baseline values (the sham group: 22.3s ± [8.0, 34.7] at baseline versus 21.7s ± [7.0, 30.0] on post-injury day seven, $p <0.01$; the TBI-only group: 41.7s ± [29.7, 66.0] at baseline versus 7.0s ± [4.0, 34.3] on post-injury day seven, $p < 0.05$). Two weeks after injury, all experimental groups showed no difference from their baseline values. Both the TBI + LPS and TBI + MINO groups showed a significant increase in latency on the rotating rod on post-injury day 14 as compared to their performance on post-injury day one (the TBI + LPS group: 13.3s ± [3.7, 23.0] on post-injury day one versus 34.0s ± [16.7,38.3] on post-injury day 14, $p<0.01$; the TBI + MINO group: 8.8s ± [2.7, 14.7] on post-injury day one versus 24.3s ± [10.3, 46.0] on post-injury day 14, $p<0.01$).

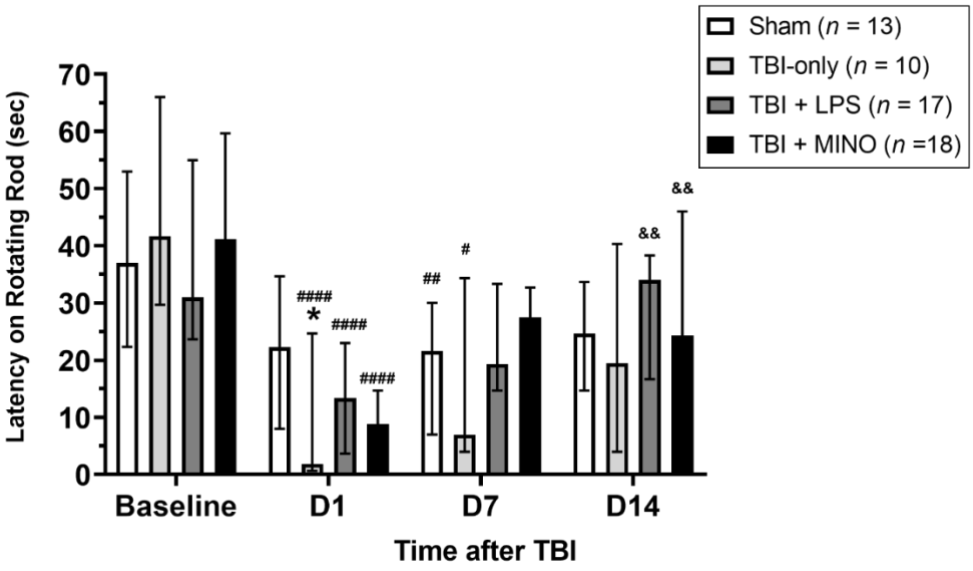

Figure 4.6 | Assessment of sensorimotor coordination and balance by using rotarod test across various post-injury time points. The TBI-only group performed worse than sham animals on post-injury day one. Both LPS and TBI ameliorated impairment in sensorimotor function at this time point. No group difference was observed on later time points. All injured animals showed worse rotarod performance than their baseline values one day after injury. Both the TBI + LPS and TBI + MINO groups showed sensorimotor recovery two weeks after injury. Data are reported as median ± 95% CI. Statistical significance: *$p < 0.05$, the TBI-only group versus the sham group; #$p<0.05$, ##$p<0.01$, ####$p<0.0001$, comparison to baseline values; &&$p < 0.01$, post-injury day 14 compared to post-injury day one. Kruskal-Wallis followed by Dunn's test for between-group comparison; Friedman test followed by Dunn's test for within-group comparison.

4.6 Both LPS and MINO resulted in impairment in recognition memory in injured rats

Hippocampal-dependent recognition memory was assessed by using the novel object recognition test. Two indices of memory were analyzed; the recognition index and the discrimination index. Recognition index was calculated as follows: (time spent with novel object in seconds /total interaction time in seconds) × 100%, where a value of 50% indicates equal time spent with both novel and familiar objects. Discrimination index was calculated as follows: (time spent with novel object – time spent with familiar object) / total interaction time, where a value of 0 indicates equal time spent with both novel and familiar objects. There were five groups included in the analysis of novel object recognition test, including naïve, sham, TBI-only, TBI + LPS, and TBI + MINO groups. A total of 62 animals underwent novel object

recognition testing. Seven animals (two from the sham group, one from the TBI + LPS group and four from the TBI + MINO group) did not explore any of the objects during the testing trial (novel object versus familiar object). Since no exploration data could be calculated, we did not include these animals in the analysis. We further excluded animals from statistical analysis for the following reasons: 1) total interaction time was less than two seconds (during one or both of the familiar and testing trials) indicating the lack of sufficient exploration (Reger et al.,2009); and 2) no approach to cereal placed in the empty chamber during all three habituation sessions indicating the lack of adequate level of habituation to the environment. Eight animals were excluded based on these criteria, including one naïve, one sham, two TBI-only, one TBI + LPS and three TBI + MINO rats. The final number of animals included in the statistical analysis was 55 rats (11 naïve, 10 sham, eight TBI-only, 15 TBI + LPS, and 11 TBI + MINO rats).

Figure 4.7 shows A) recognition index and B) discrimination index for each experimental group on post-injury day 11 as well as the performance of naïve rats. We found no group difference for either recognition or discrimination index. We observed that naïve, sham, and the TBI-only groups significantly discriminated a new object from a familiar one eleven days following injury. Both LPS and MINO resulted in impairment in recognition memory. Specifically, when comparing each group's recognition index to chance (a value of 50), the index was significantly higher than random chance in the naïve (65.8% ± 5.6%, $p < 0.05$), sham (63.3% ± 2.6%, $p < 0.001$) , and TBI-only (66.9% ± 4.7%, $p < 0.01$) groups. Both the TBI + LPS (58.6% ± 5.3%, $p > 0.05$) and TBI + MINO (59.8% ± 7.4%, $p > 0.05$) groups failed to perform significantly above chance. Similarly, when comparing discrimination index of each group to chance (a value of 0), the index was significantly higher than random chance in the naïve 0.31 ± 0.11, $p < 0.05$), sham (0.26 ± 0.05, $p<0.001$), and TBI-only groups (0.34 ± 0.09, $p < 0.01$). Both the TBI + LPS (0.17 ± 0.11, $p > 0.05$) and TBI + MINO (0.20 ± 0.15, $p > 0.05$) groups were unable to show above chance discrimination between new and familiar objects.

The total interaction time for each group during the testing trial was calculated and analyzed. We found both the TBI-only and TBI + MINO groups spent less time interacting with objects than the naïve and sham animals. Specifically, the total interaction time was significantly lower at 10.3s ± 1.8s in the TBI-only and 10.1s ± 1.9s in the TBI +MINO groups versus 20.3 ± 2.1s in naïve animals and 20.7s ± 1.5s in sham animals. Figure 4.8 displays the total interaction time for each group.

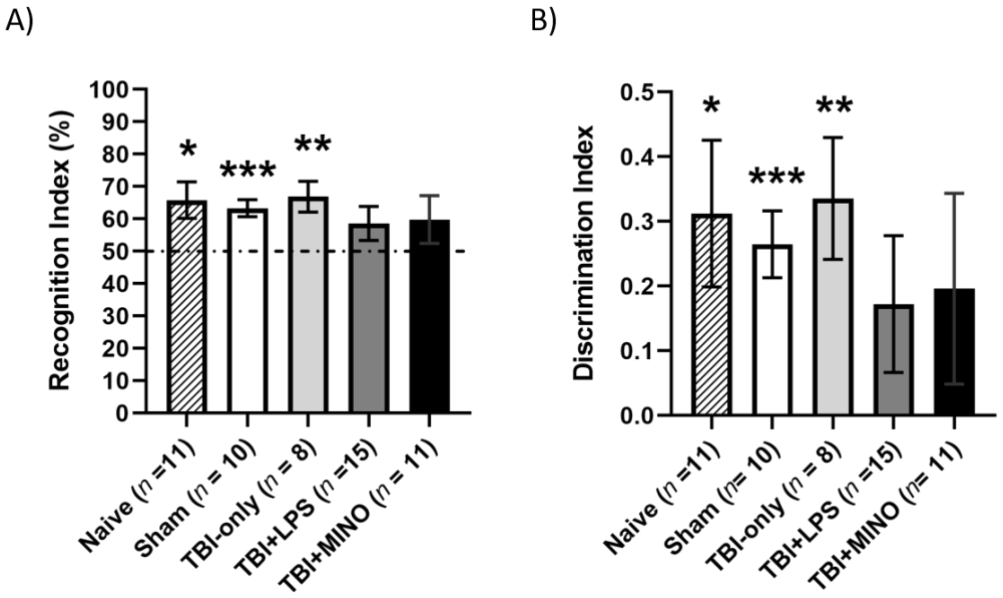

Figure 4.8 | Evaluation of recognition memory by A) recognition index and B) discrimination index in novel object recognition task on post-injury day 11. The naïve, sham and TBI-only groups significantly discriminated between novel and familiar objects. Both LPS and MINO resulted in impairment in recognition memory in the injured rats. There was no group difference observed in either recognition index or discrimination index. Data are reported as mean ± SEM. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to random chance performance: 50% for recognition index or 0 for discrimination index (one-sample t test).

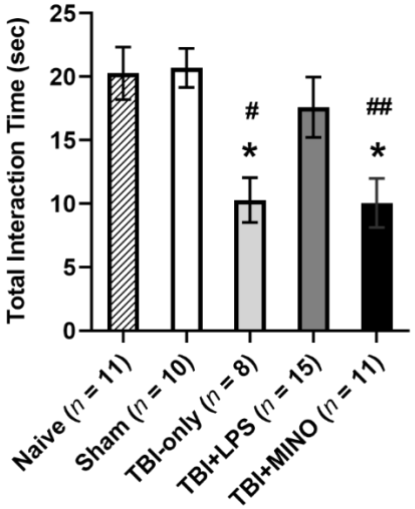

Figure 4.7 | Representation of the total interaction time in seconds for each group during testing trial in novel object recognition task. Both the TBI-only and TBI + MINO groups showed significantly reduced total interaction time compared to naïve and sham animals. Data are reported as mean ± SEM. Statistical significances: $^{*}p < 0.05$, compared to naïve animals; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to sham animals (One-way ANOVA followed by Tukey's multiple comparisons test).

4.7 Both LPS and MINO rescued spatial learning deficits following TBI

Evaluation of spatial learning was conducted by assessing learning trials in the Barnes maze on post-injury days 14 to 17. Primary latency and primary distance were used as indexes of spatial learning. Due to technical issues or errors, three rats were excluded, including one TBI-only, one TBI + LPS, and one TBI + MINO rats. Therefore, the final number was 67 rats including 12 naïve, 13 sham, nine TBI-only, 16 TBI + LPS, and 17 TBI+ MINO.

We found spatial learning deficits in injured-only rats which was not see after LPS or MINO treatment. Specifically, all groups except TBI-only showed a learning curve indicated by shorter latency as well as shorter distance travelled to locate the target hole on the last training day as compared to the first training day (i.e., post-injury day 17 versus day 14). MINO slowed down spatial learning on the last second day of training. Specifically, we found group differences in primary latency and primary distance on post-injury days 15 and 16. The TBI + MINO group (median primary latency: 32.2s ± 95% CI [20.2, 65.7]; median primary distance: 363.8 cm ± [198.8, 472.8]) showed significantly longer latency and longer distance to locate the target hole than sham animals (10.1s ± [6.1, 21.9]; 126.5cm ± [92.2,275.8]) on post-injury day 16 (Dunn's test: $p < 0.05$ for both parameters). No significant difference for primary latency or primary distance was revealed in Dunn's test on post-injury day 15. Figure 4.9 shows A) primary latency to the target hole in seconds; and B) primary distance to locate the target hole in centimetres, for each group during the learning phase.

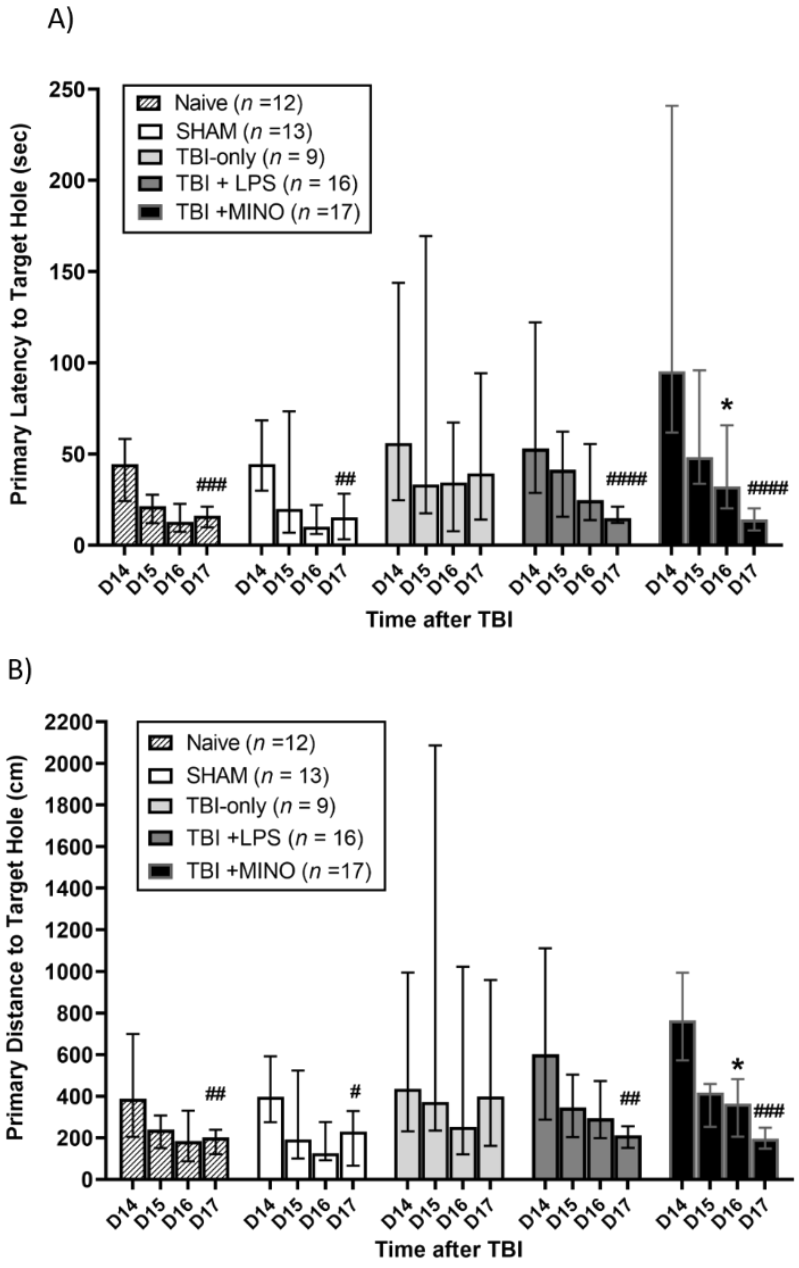

Figure 4.9 | Assessment of spatial learning by measuring A) primary latency to target hole and B) primary distance to the target hole during the learning phase of Barnes maze from post-injury days 14 to 17. Both LPS and MINO rescued spatial learning deficits seen in injured-only rats. The TBI + MINO group learned slower than sham animals on post-injury day 16. Data are reported as median ± 95% CI. Statistical significance: $*p < 0.05$, the TBI+MINO group versus the sham group; $\#p<0.05$, $\#\#p<0.01$, $\#\#\#p<0.001$, $\#\#\#\#p<0.0001$, post-injury day 17 versus day 14. Kruskal-Wallis followed by Dunn's test for between-group comparison; Friedman test followed by Dunn's test for within-group comparison.

4.8 LPS resulted in impairment in spatial memory in injured rats

After the four consecutive training days, all animals were subjected to a probe trial to evaluate spatial memory on post-injury day 18. Several indexes, including primary latency, primary distance, primary error and percent time spent in quadrant containing the target location for each group (i.e., target quadrant), were used to assess spatial memory function.

LPS resulted in deficits in spatial memory eighteen days following TBI. We did not observe such deficits in the TBI-only and TBI + MINO groups. Specifically, the TBI + LPS group (primary latency: 31.8s ± 95% CI [14.4,60.8]; primary distance: 381.6cm ± 95% CI [232.8, 585]; primary error: 22.0 ± 95% CI [8.0,33.0]) showed significant longer latency to target location and increased number of primary error than naïve animals (13.2s ± 95% CI[5.6, 18.5]; 4.0 ± 95% CI [1.0,10.0]), along with a significantly longer distance travelled to the target location than both sham (177.3cm ± 95% CI [87.6, 301.5]) and naïve groups (127.2cm ± 95% CI [68.6,241.2]). We also observed significant group differences in percent time spent in the target quadrant (Kruskal-Wallis: $p < 0.05$), but follow-up with Dunn's multiple comparisons did not reveal any specific group difference. Figure 4.10 shows A) primary latency; 2) primary distance; 3) primary error; and 4) percent time spent in the target quadrant for each group.

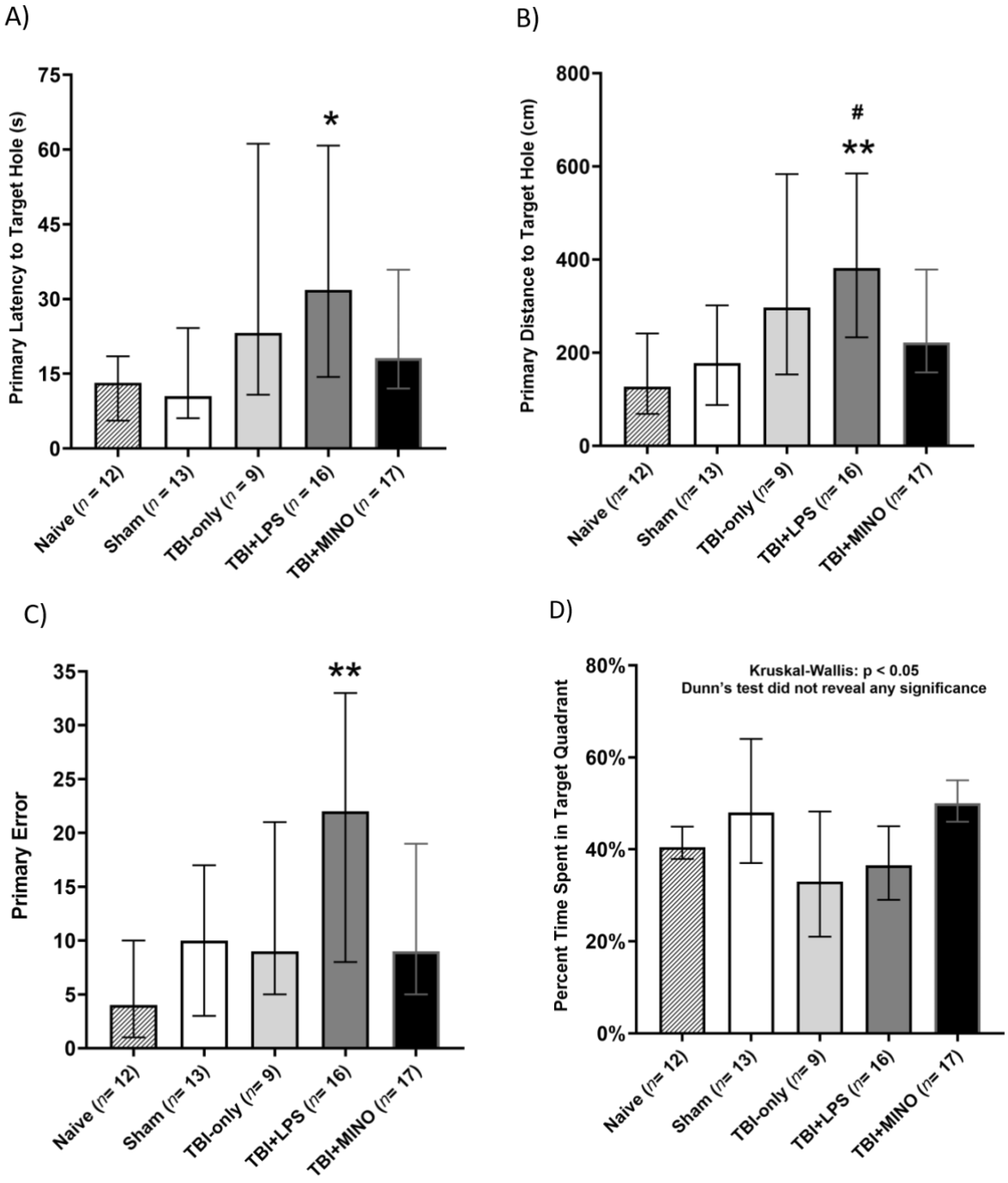

Figure 4.10 | Evaluation of spatial memory by assessing A) primary latency to the target hole, B) primary distance to the target hole, C) primary error, and D) percent time spent in the target quadrant during the probe trial in Barnes maze on post-injury day 18. LPS resulted in impairment in spatial memory in injured rats. The TBI +LPS animals had longer latency and distance to locate the target hole, as well as increased entrances to incorrect holes prior to locating the target. Data are reported as median ± 95% CI. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, the TBI+LPS group versus naïve animals; $^{\#}p<0.05$, the TBI + LPS group versus sham animals (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

4.9 Minocycline ameliorated increased seizure susceptibility nine months following TBI

A subset of animals (N = 32) were subjected to PTZ-induced seizure susceptibility testing approximately nine months after brain injury. We found an increased susceptibility to seizures induced by PTZ following FPI which was not further altered by LPS treatment. Minocycline ameliorated this increase in seizure susceptibility. Specifically, latency to the occurrence of the first seizure was 1880s ± [312, 3600] in sham animals versus 176s ± [110,831] in the TBI-only group ($p < 0.05$) and 212s ± [93, 840] in the TBI + LPS group ($p < 0.05$). The seizure duration was significantly longer in the TBI-only group (760s ± [162,2741]; $p<0.05$) and the TBI+LPS group (777s ± [33,2171], $p<0.05$) versus shams animals (25s ± [0, 843]). We did not observe a difference in seizure susceptibility in the TBI + MINO group when compared to sham animals (median latency to first seizure: the TBI +MINO group: 571s ± [249, 3600]; median duration of seizure: the TBI + MINO group:183s ± [0, 633]). Figure 4.11 displays both A) latency to first seizure in seconds and B) duration of seizure in seconds during 60 minutes after a single dose of PTZ injection.

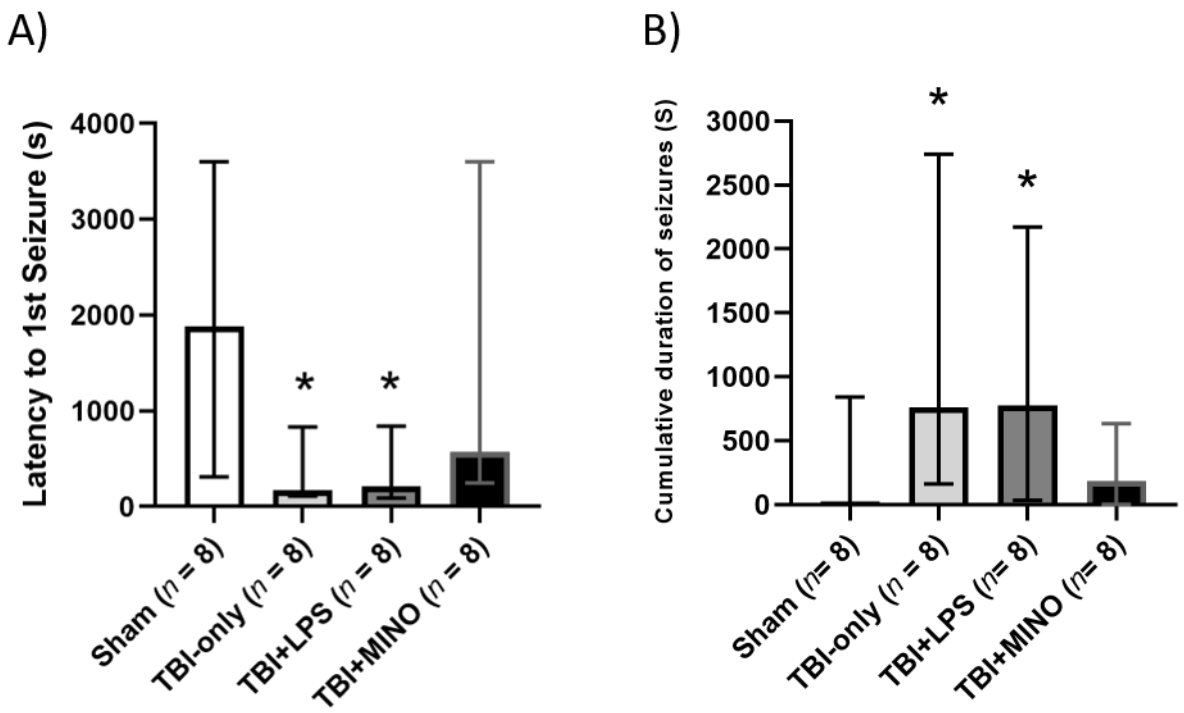

Figure 4.11 | Evaluation of PTZ-induced seizure susceptibility indicated by A) latency to the occurrence of the first seizure and B) cumulative duration of seizures at approximately nine months following traumatic brain injury. There was an increased susceptibility to seizures induced by PTZ following FPI which was not further altered by LPS treatment. Minocycline ameliorated this increase in seizure susceptibility. Data are reported as median ± 95% CI. Statistical significance: $*p < 0.05$, compared to sham animals (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

5 Discussion

5.1 Summary

In this present study, we administered LPS approximately 30 minutes prior to the induction of FPI to increase inflammation, or treated injured rats daily with MINO to reduce inflammation. The effects of increasing and decreasing TBI-related inflammation on several behavioral outcomes and seizure susceptibility were compared. The injured-only rats showed transient motor impairment, deficits in sensorimotor coordination as well as spatial learning, and an increased susceptibility to seizures, with intact recognition and spatial memory. Both LPS and MINO revealed mixed effects on these behavioral functions. Decreasing inflammation by MINO ameliorated the increased seizure susceptibility, while LPS did not alter the seizure susceptibility.

The present study is the first, to our knowledge, that directly examines the effect of minocycline or LPS-exacerbated inflammation prior to TBI in a fluid percussion injury model. There are two major novel approaches in the current study. Firstly, this study assessed the impact of drugs on multifarious outcomes following TBI, ranging from short-term motor and cognitive function to long-term seizure susceptibility. While previous studies have investigated the impact of these two drugs on several functional outcomes of TBI, their influence on the susceptibility to seizure following experimental TBI has been rarely investigated. Specifically, one paper with the pre-injury administration of LPS investigated the rate of kindled seizure acquisition in a CCI model (Eslami et al., 2005). No studies have focused on the effect of minocycline on seizure threshold. To the best of our knowledge, the most closely related study used an inhibitor of proinflammatory cytokine upregulation named Minozac and investigated its effect on susceptibility to seizure induced by electroconvulsive shock (Chrzaszcz et al., 2010).

The second novel aspect of the present study lies in the use of a mixed focal and diffuse injury model, the FPI (Thompson et al., 2005). All related studies involved different experimental models, such as CCI (LPS studies: Longhi et al., 2011; Eslami et al., 2015; MINO studies: Sanchez Mejia et al., 2001; Adembri et al.,2014), weight-drop impact acceleration (LPS: Collins et al., 2018; Hang et al., 2004; MINO: Homsi et al., 2009; Homsi et al., 2010), midline FPI (LPS: Muccigrosso et al., 2016), and blast (MINO: Kovesdi et al., 2012). Although experimental TBI models generally replicate key clinical features, distinct injury mechanisms and different levels of severity exist among various models. While the CCI model produces a

mainly focal type of injury (Gold et al., 2013), the FPI model produces a more diffuse injury on both white matter and grey matter (Johnson et al., 2015). Even within the FPI models, the specific location of craniotomy defines the sub-types of injury (Ziebell et al., 2016; Floyd et al., 2002; Vink et al., 2001). Some of these LPS studies were conducted in MFPI, which was a milder injured compared to parasagittal or lateral FPI (Muccigrosso et al., 2016; Hicks et al., 1996; Bramlett &Dietrich, 2002; Smith et al., 1997). Moreover, the current study induced moderate to severe TBI using the FPI model, which produces more severe injury than the mild blast injury and closed head injury weight drop model used in previous related studies (Kovesdi et al., 2012; Siopi et al., 2012).

Together, these innovations in the current study aimed to obtain a better understanding of the role of TBI-induced inflammation in multiple consequences following the initial injury. In the following sections, the effects of FPI, LPS, and minocycline on various post-injury outcomes in this study are discussed. It is important to note that no difference in the initial severity assessed by righting reflex was observed among injured groups, implying that any difference in the outcome observed between injured groups was not due to a difference in the initial injury impact.

5.2 The Effect of FPI

5.2.1 Impact on Behavioral Outcome

The current study characterized the FPI model on multiple behavioral outcomes. The induction of FPI selectively resulted in impairment in certain types of behavioral function, including transient neurological motor impairment, dysfunction of sensorimotor coordination, and deficit in spatial learning, with intact recognition and spatial memory.

As seen in previous studies using the FPI model in rats, the TBI-only rats in the current study exhibited neurological motor deficits, indicated by lower composite neuroscores than sham animals (Doll et al., 2009; Maegele et al., 2005; Zhang et al., 2005). The neurological motor deficit was transient in injured-only rats and they recovered to the same level as sham rats by post-injury day 15. They remained no different from sham animals at post-injury day 28. Some previous FPI studies observed more persistent neurological motor deficits for up to 12 weeks post injury (Zhang et al., 2005). One possible reason for the discrepancy could be a difference in

the severity of injury between studies. We induced less severe injury (2.0 to 2.5 atm) than was used (2.8 – 3.3 atm) in a previous study (Zhang et al., 2005). A previous study showed that a small change in craniotomy position in the FPI model can also affect motor outcome as assessed by composite neurological score (Vink et al., 2001). If the FPI model had a craniotomy that was more than 3.5 mm from midline, an increase in motor deficit was seen as compared to those with a location less than 3.3 mm from midline (Vink et al., 2001). In the current study, a less lateral craniotomy (2.5 mm from midline vs more than 3.5 mm) was used, which may contribute to the less persistent motor deficit we observed.

The impairment in sensorimotor coordination was also transient in injured rats. This deficit was only detectable one day after TBI, with spontaneous recovery at one- and two-weeks following injury. This is consistent with a previous study that used a similar craniotomy location and injury severity as the current study (AP 3.8mm, ML 2.5mm, 1.9 ± 0.2 atm). In the previous study impairment in the rotarod test was only reported one day after injury (Chen et al., 2015). It is also worth mentioning that Hamm and colleagues tested the sensitivity of the rotarod test in the FPI model and found motor deficits up to 5 days (Hamm et al., 1994). We did not evaluate the performance on the rotarod test between post-injury days one and seven. Injured-only rats might have changes in the sensorimotor function between these two time points and differences compared to sham animals might be detected if another time point within these time windows was conducted. Other studies with moderate FPI have used a higher acceleration rate (5rpm/10sec versus current study: 3rpm/10sec) in the rotarod test. These authors have discerned sensorimotor dysfunction for a longer period of time (i.e., weeks after injury) (Doll et al., 2009; Riess et al., 2007). It would seem likely that higher acceleration rates may be more amenable to the detection of small differences between sham and injured animals.

Spatial learning deficits after the induction of FPI were evident in the current study, consistent with previous findings that FPI can impair the learning performance on Barnes maze (Doll et al., 2009; Lee et al., 2013). No impairment in spatial memory was observed after TBI. This suggests that the function of spatial learning rather than spatial memory recall may be susceptible to the to the damage induced by FPI within three weeks after injury. It is also possible that motor dysfunction might contribute to learning deficits seen in injured rats, since motor function was not measured during the same testing time points as the Barnes maze. Affective functions such

as anxiety and depressive-like behaviors may also influence an animal's performance in a cognitive test. The lack of corresponding behavioral tests in this present study cannot rule out such possibility.

We found intact object recognition memory at 11 days post-TBI. This finding contrasts with some other TBI studies which reported impairment in a NOR task (Chen et al., 2015; Huang et al., 2014; Prins et al., 2010). A few factors may explain the conflicting results. Firstly, the severity of FPI can be a key factor for detecting cognitive function. A study reported impaired performance in NOR in their "high" fluid perfusion group (6.0 ± 0.5 atm), but not in the "low" group (1.9 ± 0.2 atm) (Huang et al., 2014). In another similar study, "low" FPI rats had deficits in NOR in the first week after TBI and recovered to the same level as sham by the second week (Chen et al., 2015). The impairment was continuously observed in "high" FPI group up to eight weeks in both studies (Chen et al., 2015; Huang et al., 2014). Based on these findings, rats with a "low" injury level may show more obvious impairment in the NOR test in the first week after FPI and the deficit can diminish by the second week. The injured rats in the present study (2.0 to 2.5 atm) were comparable to the "low" group in these studies (1.9 ± 0.2 atm). Since the testing time point in the current study (post-injury day 11) was beyond one week and approaching to post-injury week two, the intact recognition memory replicated the findings in the "low" group in these previous studies. The interval between the familiar and testing trials may also play a role in detecting the deficits in this behavioral test. For instance, juvenile Sprague Dawley rats (postnatal day 35) showed impairment in the NOR test after a single mild CCI only when the intertrial period was 24 hours but not one hour (Prins et al., 2010). It is important to mention the recognition index of naïve rats in the present study was around 65%, which replicates what has been reported in male adult Sprague Dawley rats (Reger et al., 2009), showing an effective experiment paradigm.

5.2.2 Impact on Seizure Susceptibility

We observed an increased susceptibility to PTZ-induced seizures at approximately nine months post-injury. This result is consistent with previous reports that FPI results in an increased seizure susceptibility (Atkins et al., 2010; Bao et al., 2011; Kharatishvili et al., 2006, 2007).

5.2.3 Impact on Cytokine Expression

The present study identified the concentration of cytokines and chemokines, including IL-1ß and MIP-1α (CCL3), was increased at six hours after the induction of FPI, consistent with previous reports showing rapid upregulation of cytokines and chemokines after TBI (Knoblach & Faden, 2000; Mukherjee et al., 2011). Observation of elevated levels of a pro-inflammatory cytokine and a chemokine confirmed the production of key inflammatory mediators as well as the involvement of recruiting leukocytes from the peripheral system, which are among the main characteristics of neuroinflammation following TBI (Chiu et al., 2016; Morganti-Kossmann et al., 2001). Other cytokines such as TNFα and IL-10 may have a peak time earlier than the six-hour assessment point, as has been seen in other studies (Fan et al., 1996; Knoblach & Faden, 1998). On post-injury day seven, all cytokine levels decreased and no differences were found in injured animal compared with shams, consistent with a previous report by Dalgard and colleagues (Dalgard et al., 2012).

5.3 The effects of LPS

When evaluating the acute inflammatory response in TBI rats injected with LPS, the concentrations of IL-1ß and MIP-1α /CCL3 were upregulated at six hours post-injury as seen in injured-only rats, indicating a similar acute injury-related inflammation response. Although the administration of LPS did not result in an increase in cytokine levels compared to injured-only rats, some differences were observed compared to sham that were not found in the TBI-only group. When compared to sham animals, LPS resulted in higher concentrations of MCP-1/CCL2 and RANTES /CCL5 at six hours post-injury, which was not seen in injured-only rats. The elevated concentrations of chemokines support the notion that a peripheral inflammation insult can promote neuroinflammation, partially through increasing recruitment of peripheral immune cells, such as monocytes (Collins-Praino et al., 2018; Gyoneva & Ransohoff, 2015).

Increased levels of cytokine expression compared to the injured-only rats were not observed partially suggesting that the neuroinflammation response in the context of TBI may have a maximum effect that it can reach. It was also possible that we did not capture the peak production of cytokines and chemokines by LPS in the injured rats. A study using a similar dosage reported the production of cytokines within 3 hours after the administration, with no change in levels seen by 24 hours in uninjured animals (Teeling et al., 2007). Since we only

evaluated the inflammatory response acutely at six hours post-injury, it is uncertain if the inflammatory response would be altered or not at other time points. We might have missed the peak inflammatory response that could have happened before six hours post-injury. Moreover, the injection time of LPS (approximately 30 minutes prior to the induction of injury) may not result in a peak inflammatory response yet at the time of injury, which was typically one hour or more after the systemic injection, as seen in previous studies (Kakizaki et al., 1999; Murray et al., 2012). However, it is not clear how the timing of the peak inflammatory response in relation to injury would actually alter the outcome.

The first hypothesis regarding the augmented effect of LPS on behavioral deficits was partially supported. Upregulating inflammation by LPS administration resulted in more severe deficits in specific neurological functions within one month following TBI as compared to injured-only rats. Injured rats injected with LPS showed slower neurological motor recovery, deficits in recognition memory, as well as impairment of spatial memory recall that were not observed after TBI alone. In contrast, the administration of LPS displayed a positive impact on certain outcomes. Unlike the injured-only rats, the post-injury functions of sensorimotor coordination and spatial learning were preserved in TBI rats injected with LPS. The second hypothesis regarding the augmented effect of LPS on seizure susceptibility was not supported. The administration of LPS did not further increase seizure susceptibility compared to injured-only rats approximately nine months after injury. Overall, while the injection of LPS 30 minutes before the injury did not aggravate seizure susceptibility, it did reveal dual effects in motor and cognitive functions.

In comparison with other TBI studies utilizing LPS, the current study showed some results similar to studies with LPS preconditioning as well as those with post-injury LPS administration. For example, preconditioning with LPS in mice five days prior to the induction of CCI also led to better performance than in TBI mice treated with saline in another vestibulomotor test, the beam walking test (Longhi et al., 2011). The same study also reported better performance in composite neuroscore, which was opposite to the current findings (Longhi et al., 2011). Regarding performance in the Barnes maze, the current study partially replicated findings from studies injecting LPS post-injury. Impairment in the probe phase was also observed in studies injecting LPS at five days following mild weight drop impact-acceleration

injury in rats (Collins-Praino et al., 2018) or at 30 days after midline FPI in mice (Muccigrosso et al., 2016). Deficits during the learning phase in the Barnes maze were also found in these two studies, which was not replicated in the current study. The beneficial effect of LPS in preventing the acceleration of kindling as seen in a previous study was not observed as a reduction in PTZ seizure susceptibility in the current study (Eslami et al., 2015).

It is essential to draw the distinction that previous TBI studies involving LPS represent different phenomena than the current study. Studies of preconditioning with LPS found beneficial effects on many behavioral outcomes and reported the prevention of accelerated kindling observed in injured rats (Longhi et al., 2011; Eslami et al., 2015). Such a preconditioning phenomenon requires time, usually days after administration, to develop and prime microglia phenotypes that ultimately leads to a protective effect in response to a subsequent brain insult (Longhi et al., 2011). Other studies using LPS to induce peripheral immune challenge days (five or 30 days) after TBI, showing exacerbation of functional deficits, neuronal damage and microglial activation following the immune challenge (Collins-Praino et al., 2018; Corrigan et al., 2017; Muccigrosso et al., 2016). These findings prove a persistent TBI-induced inflammation response and confirm that a peripheral inflammatory insult secondary to initial brain injury worsens outcomes. In contrast, the administration of LPS in the current study was neither days before injury for a preconditioning effect, nor days after injury for a secondary insult to an already injured brain. Instead, in the current study, the peripheral immune insult was introduced to simultaneously interplay with TBI-induced inflammation and examine whether it altered the outcome following TBI. The mixed effects of LPS observed in the current study appear to be something between the findings of these two types of studies, suggesting the timing for modulating the TBI-related inflammation response is a crucial factor influencing the outcomes following TBI.

The upregulation of inflammation by the administration of LPS 30 minutes prior to the induction of injury did not further increase the post-injury acute and subacute inflammatory responses compared to injured-only animals, but some differences were observed compared to sham animals that were not seen in injured-only rats. The LPS administration was detrimental to hippocampal-dependent memory functions, but also prevented the loss of post-injury vestibulomotor and spatial learning functions seen after injury alone. The susceptibility to

seizures after injury was not altered by LPS. These mixed effects of LPS imply that an additional immune challenge to the robust TBI-induced inflammatory response does not worsen all of the consequences following TBI. A ceiling effect of the neuroinflammatory response to CNS insults might be one possible explanation. This study also suggests a beneficial role of acute TBI-related inflammation with the purpose of repairing the injured tissues (Woodcock & Morganti-Kossmann, 2013).

5.4 The effect of Minocycline

The first hypothesis regarding the effect of minocycline on behavioral outcome was partially supported. Similar to the mixed effects of LPS in the short-term, the minocycline treatment also revealed differential effects on post-injury behavioral outcomes. Minocycline treatment did not alter neurological motor function in injured rats, showing no difference in composite neuroscores compared to the TBI-only group at all testing time points. In contrast, minocycline treatment ameliorated the impairment on the rotarod task at one day post-injury, the same outcome observed with administration of LPS. Similar results were shown in a previous study which reported improved performance on the beam walk task at post-injury day one with minocycline treatment, with an absence of better neurological recovery following CHI (Bye et al., 2007). Additionally, the current findings confirm the results in a study that reported no difference in performance in rotarod task between injured-only and minocycline-treated rats at seven to 11 days post-CCI (Vonder Haar et al., 2014). Regarding post-injury cognitive functions, minocycline was able to rescue the loss of spatial learning ability but resulted in a recognition memory deficit that was not seen in injured-only rats at 11 days after TBI (Siopi et al., 2012). Unlike LPS, minocycline did not alter the intact spatial memory in injured rats. The findings in NOR performance contrast with a previous study which reported minocycline attenuated the memory impairment up to 13 weeks post-CHI in mice (Siopi et al., 2012). This previous study tested the effect of minocycline on the NOR task at a much later time point (i.e., four weeks post-CHI) than the current study, suggesting that the beneficial effect of minocycline may be elicited in a more chronic phase. This impairment in performance in the NOR task also resulted from the administration of LPS, which may indicate this specific type of memory is sensitive to inflammation status following TBI. Deviating from an optimal level of inflammation in either direction may be deleterious for injured brains, possibly resulting in impairment of the preservation of recognition memory. While intact spatial memory was

observed in both injured-only and minocycline-treated rats, minocycline successfully protected spatial learning that was disrupted after TBI. This neuroprotective effect in spatial learning measured by the Barnes maze was also reported in a study using a rat blast model (Kovesdi et al., 2012). Furthermore, the same protective effect was observed in rats injected with LPS, suggesting that the function of spatial learning is easily affected by the modulation of the inflammatory response.

The second hypothesis regarding the beneficial effect of minocycline on reducing seizure susceptibility was supported. Minocycline treatment significantly ameliorated the increase in seizure susceptibility observed nine months after the induction of FPI. This finding is essential because minocycline treatment in a subacute period successfully ameliorated increased seizure susceptibility in the long-term following TBI. This finding is in agreement with a previous study which reported the prevention of increased seizure susceptibility by MINOZAC (i.e., a suppressor of proinflammatory cytokine production) treatment in a mouse midline closed skull TBI (Chrzaszcz et al., 2010).

Within six hours following TBI, the inflammatory response of minocycline-treated rats was similar to the profile seen in injured-only and LPS-injected rats. However, the increased level of IL-1ß in the cortex of injured-only and LPS-injected rats was not seen in the minocycline-treated group, which supports the previous finding by Bye and colleagues (Bye et la., 2007). A week after TBI, decreased expression of MIP-1α / CCL3 and RANTES/CCL5 in the hippocampus was detected in minocycline-treated rats. CCL3, CCL5 and their shared receptor CCR5 have been reported to be elevated in epilepsy (Galasso et al., 1998; Mennicken et al., 2002; Van Gassen et al., 2008). A study found that seizures induced by kainic acid resulted in increased expression of both CCL3 and CCL5 (Louboutin et al., 2011). Decreasing the expression of CCR5 strongly protected rats from induced seizures (Louboutin et al., 2011). Therefore, reduction of CCL3 and CCL5 production may potentially represent a mechanism for the attenuation of increased seizure susceptibility with daily minocycline treatment in the current study.

It is imperative to acknowledge that the injection paradigms between TBI studies using minocycline are varied. Most studies started the first dosage within one hour after the induction of TBI, which also occurred in the present study (Homsi et al., 2009, 2010; Siopi et al., 2012;

Ng et al., 2012; Chhor et al., 2017). A common injection paradigm consisting of repetitive injection every 12 hours usually for three to four consecutive days and up to two weeks was applied (Vonder Haar et al., 2014; Sanchez Mejia et al., 2001; Hanlon et al., 2016; Lam et al., 2013; Ng et al., 2012). To maximize the anti-inflammatory response, some studies shortened the injection interval to every three hours for a total of three injections (Siopi et al., 2012; Homsi et al., 2010). All of these studies focused more on measuring outcomes in a shorter period of time, usually within one month following TBI. The current study modified the above-mentioned injection paradigms with a prolonged injection period. The chronic treatment extending beyond two weeks may be a key to amelioration of deleterious outcomes that occurred months later (i.e., the reduced seizure threshold). We observed that a few outcomes were exacerbated by minocycline in this study, suggesting that the chronic delivery of minocycline could also possibly suppress a beneficial component of TBI-induced inflammation (Bye et al., 2007). Based on the current results, it is unclear what might be the mediators attributing to the lack of protection against neurological recovery and recognition memory.

The inconsistent effects of minocycline and LPS suggest that inflammation can be beneficial as well as detrimental, depending on the condition and the timing after TBI. Perhaps, targeting the inflammation is not adequate to address the ongoing and dynamic secondary injury cascades following TBI. Considering there are various mechanisms involved in the secondary injury, it would be important to target other factors in these complex neuropathologies. There are more recent studies targeting both inflammation and oxidative stress following TBI (Sangobowale et al., 2018 a, b; Abdel Baki et al., 2010; Haber et al., 2013). In these studies, minocycline was combined with *N*-acetylcysteine (NAC) in both CCI and CHI models. Minocycline plus NAC limited both cognitive and memory deficits after mild CHI (Haber et al., 2013). Positive effects of the combined drugs remain potent, even when the first dosage starting 12 hours after TBI (Sangobowale et al., 2018 a, b). These findings suggest that a multimodal approach yields more powerful therapeutic effects than targeting inflammation using minocycline alone.

In summary, minocycline treatment in the subacute period following TBI successfully ameliorated the increased seizure susceptibility seen many months after the initial injury. In the short term following TBI, minocycline treatment showed a transient positive effect on sensorimotor function, but resulted in impairment in recognition memory.

5.5 Limitations

The present study has several limitations. First, a large amount of variability was observed in our data. In part, this could have arisen because the injury induced in this FPI model replicates the heterogenous range of injury seen human TBI. Another factor could be that some rats went on to develop post-traumatic epilepsy while others did not. Behavioral deficits such as spatial learning impairment can be induced by the epileptic state (Detour et al., 2005; Mortazavi et al., 2005). However, the current study did not investigate this possibility.

Second, there were no other control groups to compare the effects of two drugs. Since the minocycline treated animals were injected repetitively, a control group of injured rats treated with daily saline would have taken the effect of repetitive injection into consideration, resulting in more comprehensive comparisons. There was also no control group that addressed the impact of injecting LPS to sham animals in the current study. Adding these controls groups would have significantly increased the number of animals used, which is a major ethical consideration, and it also would not have been feasible to complete the testing in this large number of animals for this thesis.

Third, the evaluation of behavioral outcome in the current study was conducted within one month following TBI. Thus, the impact of both LPS and minocycline on those behavioral functions in a longer time period following TBI cannot be concluded.

Fourth, we only examined a limited number of inflammatory mediators at two time points. The two time points were far from each other; thus, we cannot exclude the following possibilities: 1) the involvement of other cytokines and chemokines in the effects observed in this study; and 2) any changes of cytokines at other time points.

Last, but not least, minocycline was injected right after the rat regained its righting reflex, which was within 30 minutes after the induction of TBI. Injection at this relatively short time after TBI is hard to achieve in clinical cases. This injection timing may fail to represent a clinically relevant time window in human TBI, adding challenges to interpret or even translate such findings to the clinical setting.

5.6 Conclusion

The current study investigated the effects of LPS and minocycline on the post-injury inflammatory response, behavioral outcomes and seizure susceptibility following the induction of fluid percussion injury in rats. LPS did not alter initial injury magnitude or augment post-injury expression of CNS cytokines. Minocycline reduced the subacute expression of specific chemokines CCL3 and CCL5 compared to injured-only rats. Both LPS and minocycline had mixed effects on behavioral function and recovery during the first post-injury month. The administration of LPS slowed the neurological recovery pattern in injured rats, increasing impairment in object recognition and spatial memory. While both LPS and minocycline provided transient protection against sensorimotor dysfunction, minocycline also significantly prevented the increased seizure susceptibility. Minocycline treatment also led to deficits in recognition memory. These findings suggest that daily minocycline treatment in the subacute period after TBI may lead to lasting protection against increasing seizure susceptibility. Mixed effects of LPS and MINO suggest that simply supressing or augmenting the inflammatory response may fail to address the complexity of secondary injury after TBI.

6 Future Directions

6.1 Does inflammation impact the occurrence of spontaneous seizures after FPI?

The current study utilized PTZ to examine seizure susceptibility following TBI. The higher seizure susceptibility shown in a PTZ test can only tell us about the presence of hyperexcitability, but not the development of PTE. Post-injury spontaneous seizures occur without the induction of chemicals. To identify changes in the development of PTE, we will need to examine this hallmark, the occurrence of spontaneous seizures. In our lab, ongoing work has been done to examine whether LPS and MINO will have impact on the development of PTE.

Previous studies have found FPI-induced spontaneous late seizures. For example, Reid and co-workers have demonstrated that spontaneous focal seizures occurred in 50% of LFPI rats (Reid et al., 2016). Focal seizures started anterior to or at the injury site, with an increase in seizure duration and frequency over time (Reid et al., 2016). An earlier study found that 43% to 50% of rats developed spontaneous late seizures during a 12-month follow up after severe FPI (Kharatishvili et al., 2006). When monitoring moderate FPI rats for three consecutive weeks, spontaneous seizures were not observed at 11 months post-injury, although 80% of FPI rats had spiking, an indication of hyperexcitability (Kharatishvili et al., 2007). The proposed future study will characterize the occurrence of spontaneous seizures after FPI and examine whether inflammation influences this outcome.

Rats in all experimental groups have been implanted with multiple electrodes at approximately four months post-injury, as described in the methods section of this thesis. Long-term continuous EEG monitoring was performed to capture the occurrence of spontaneous seizures. We have monitored rats with video-EEG beginning four months post-injury for four-five days continuously, at repeated intervals of every four weeks. Each EEG file will be analyzed to detect the occurrence of epileptiform activity, spontaneous seizures or spiking. Seizures are defined as at least 5 s duration of high-amplitude rhythmic discharges that show a clear onset, temporal changes in wave morphology and amplitude, and offset (Kharatishvili et al., 2007). A spike is defined as a high amplitude and sharply countered waveform that lasts 20 to 70 ms (Kharatishvili et al., 2007).

The number of spikes and seizures during the entire period of monitoring will be measured for each rat. Rats that display at least two spontaneous seizures will be considered epileptic (Kharatishvili et al., 2006). In animals with PTE, the latency period to first seizure, frequency of occurrence of spontaneous seizures, and duration of each seizure will be analyzed. Behavioral severity for each seizure will be also scored utilizing a modified Racine type scale by reviewing the corresponding video (Racine, 1972). The scoring paradigm will be as follows: score 0 = freezing; score 1 = movements of mouth and face, head nodding; score 2 = clonic jerks of one forelimb; score 3 = bilateral forelimb clonus; score 4 = forelimb clonus with rearing; score 5 = forelimb clonus and rearing with falling (Kharatishvili et al., 2006).

This study aims to answer the following:

1. Does increasing inflammation with LPS augment the development of PTE in injured rats? If so, what characteristics of PTE are changed? Although LPS did not alter seizure susceptibility, it is possible that LPS will influence the occurrence of spontaneous seizures. We expect that LPS will augment the development of PTE as indicated by more spikes, a shorter latency period to develop seizures, more frequent seizures, and longer duration of seizures as compared to injured-only rats.

2. Does minocycline treatment help reduce the development of PTE in injured rats? If so, what characteristics of PTE are changed? We have demonstrated minocycline prevents the increase in seizure susceptibility seen in injured rats and expect the same effect on spontaneous seizures. We anticipate minocycline will ameliorate the development of PTE, as evidenced by a reduced number of spikes, a longer latency period, and less frequent seizures and shorter duration of seizures as compared to injured-only rats.

6.2 Does inflammation impact behavioral changes in the chronic phase?

Animals in our study were subjected to behavioral testing including motor and cognitive function within one month after FPI. There are two limitations associated with this study design. First, there is a lack of behavioral assessment in the chronic post-injury phase. We cannot conclude how inflammation might influence these behaviors in a longer period following TBI.

Second, we did not conduct affective behavioral testing, such as anxiety- and depressive-like behaviors. We cannot rule out the effect of affective factors in behavioral outcomes. To address these two concerns, an experiment consisting of motor, cognitive and affective functional assessment at more than one month post-injury will be needed. First, the testing time point will be conducted at three months post-injury, which has been done in previous TBI studies (Corrigan et al., 2017; Lyndsey et al., 2018). Second, elevated plus maze and forced swim tests will be added to the chronic behavioral testing paradigm. The elevated plus maze is commonly used to measure anxiety in rodents (Walf & Frye, 2007). In this test, rats will be given five minutes to explore freely an elevated cross-shaped maze consisting of two closed and two open arms (Corrigan et al., 2017). Preference of closed arms over the open arms is an indication of anxious behaviors (Corrigan et al., 2017). Increased anxiety using this task in rodents after FPI has been observed (Semple et al., 2019). This deficit can last more than one month following TBI (Shultz et al., 2012, 2013). The forced swim test assesses depressive-like behavior (Bogdanova et al., 2013). Rats are placed in a cylinder filled with water at a depth of 30 cm for six minutes (Corrigan et al., 2017). Despair and helpless behaviors are observed when a rat floats and becomes immobile (Bogdanova et al., 2013; Corrigan et al., 2017). Depressive-like behavior has been demonstrated to persist more than one month after FPI (Shultz et al., 2012, 2019).

A behavioral battery will be performed in all experimental groups of animals at three months post-injury, including 1) composite neuroscore (day 90); 2) rotarod (day 91); 3) elevated plus maze (day 92); 4) Barnes maze (day 93 to 96); and 5) forced swim test (day 97). The order of testing is based on stress levels, starting from the least to the most stressful test (Corrigan et al., 2017).

This experiment should help us answer the following:

1. Does increasing inflammation with LPS aggravate motor, emotional, and cognitive deficits in injured rats three months post-injury? We have found LPS aggravated memory deficits within one month after TBI. In the longer term, the detrimental effect of LPS will possibly be stronger. We expect that the TBI + LPS group will show worse performance in motor, emotional, and cognitive tasks compared to injured-only rats three months post-injury.

2. Does minocycline treatment lead to milder motor, emotional, and cognitive deficits in injured rats three months post-injury? We have demonstrated that minocycline treatment provided transient protection against motor deficits while leading to recognition memory impairment within one-month after TBI. The positive effect of minocycline might be more robust in a chronic phase. We expect that minocycline treatment will lead to milder deficits in motor, emotional, and cognitive tasks compared to injured-only rats three months post-injury.

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