Impact of an Early Life Immune Challenge on Outcomes in a Rat Model of Pediatric Mild Traumatic Brain Injury

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Impact of an Early Life Immune Challenge on Outcomes in a Rat Model of Pediatric Mild Traumatic Brain Injury

by

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Abstract

The focus of this thesis was to determine if neonatal exposure to lipopolysaccharide (LPS) on postnatal day (P) 10 would affect the behavioural and molecular outcomes after mild traumatic brain injury (mTBI) on P30. It appears that female LPS + mTBI rats show alterations in measures of anxiety, working memory and depressive-like behaviour - different from those not previously exposed to LPS. In addition to behavioural changes, the mRNA expression profiles of LPS + mTBI rats showed a differential cytokine response at P45 in comparison to rats that received LPS, mTBI, or saline injury controls (SAL + mTBI). Cytokine mRNA levels were followed up by analyzing two microglia/macrophage phenotype markers; the markers also showed an altered profile for LPS + mTBI animals. The findings of the work highlight the importance of sex differences and suggest that an early life immune challenge could confer selective susceptibility to outcomes after mTBI.
Acknowledgements

I would like to acknowledge my supervisor and committee members Dr. Michael Esser, Dr. Quentin Pittman, and Dr. Richelle Mychasiuk who all gave incredibly valuable input, knowledge, and told me to stop thinking of future projects and finish this one. I would like to thank the incredible people in HMRB 233 and the Pittman Lab: especially Harleen Hehar, Irene Ma and Katrina Yu for not only providing an incentive to go into the lab on mornings and weekends but also providing lots of laughs along the way. Thanks to my amazing family, who didn’t understand the project, but nodded and smiled at me when I told them about it! Both my friend Blythe and I have experienced a mTBI and only she went on to develop PCS. I guess you drew the short straw...thanks for making this research project personal. Finally, a big acknowledgement to Megan Lewis, who took me on as a lab volunteer and not only showed me that research can be enthralling but opened up a whole lot of doors along the way. Thank you!
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<thead>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled cortical impact</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase two</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHI</td>
<td>Closed head injury</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DHPC</td>
<td>Dorsal hippocampus</td>
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<tr>
<td>FPI</td>
<td>Fluid percussion injury</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GSC</td>
<td>Glasgow coma scale</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary-adrenal</td>
</tr>
<tr>
<td>HYPO</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICD</td>
<td>International classification of disease</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LOC</td>
<td>Loss of consciousness</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mTBI</td>
<td>Mild traumatic brain injury</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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Chapter One: Modulation by neuroinflammation: Examining the outcome of a neonatal lipopolysaccharide exposure on a rat model of mild traumatic brain injury

1.1 Introduction to mild traumatic brain injury (mTBI)

1.1.1 Epidemiology

The number of people affected by mild traumatic brain injury (mTBI) is difficult to ascertain due to variations in diagnostic criteria. Estimates from the Center for Disease Control and Prevention in the United States report over 1.7 million people present to the emergency department or are hospitalized with mTBI annually (Laker, 2011). The variation in the statistics reported may be caused by an unclear definition of mTBI. Some studies have looked at loss of consciousness (LOC) for less than thirty minutes, others at Glasgow Coma Scale (GCS) ratings from 13-15, or an International Classification for Disease (ICD) 9 code of 850 (Cassidy et al., 2004). Besides various diagnostic criteria, there is also some confusion between the terms mTBI and concussion. Simply put, mTBI is defined as “an alteration in brain function or pathology caused by an external force” (Menon et al., 2010) whereas a concussion is defined as “a complex pathophysiological process affecting the brain and is induced by traumatic biomechanical forces” (McCrory et al., 2009). A panel of experts included common features in the definition of concussion such as rapid onset and a greater degree of functional disturbance rather than structural (including no difference in structural imaging) (McCrory et al., 2009).

In addition to the difficulty of an inconsistent definition, the incidence of concussion and mTBI might be over-reported with increased recent media attention for high profile injuries (Robbins et al., 2014). Although mTBI might be over-reported by some groups there is also evidence that some people don’t report the injury at all. Most cases of mTBI are recorded when
the person has been taken to hospital and received treatment, but in a self-report study as many as 25% of people who experienced a mTBI do not seek medical care (Sosin et al., 1996). The difficulty in a clear definition and presentation could be partly due to the heterogeneous symptoms that present across individuals. Many people experience motor, social, and cognitive impairments following mTBI. These include symptoms such as: headaches, dizziness, memory deficits, difficulty concentrating, fatigue, anxiety, and depression (Alexander, 1995).

Although not life-threatening, these symptoms can become serious and debilitating; they are also quite common as mTBI accounts for approximately 70-90% of brain injuries treated (Cassidy et al., 2004). An age group that have been shown to be at high risk for suffering mTBI is that of young children and adolescents (Barlow et al., 2015, 2010). The severity of the impairment and recovery time varies between individuals but in the case of adolescents, approximately 10 - 15% of those who received a mTBI were still experiencing symptoms three months after the initial injury (Barlow et al., 2010). These persistent symptoms that can last months or even years are collectively known as post concussive syndrome (PCS) (Ryan and Warden, 2003). While it is currently unknown what contributes to recovery (‘resilient’) in individuals versus those that develop PCS (‘susceptible’), several factors have been suggested to be modifiers of outcome and include: age and sex of the patient, the duration of the loss of consciousness, psychosocial factors before and following injury, and pre-existing neuropathological conditions (Ryan and Warden, 2003). For those 10 – 15% of children who experience lingering symptoms, it can have consequences on their neuropsychological development and academic performance at school (Kirkwood et al., 2006).
In addition to persistent symptoms for individuals that develop PCS there are high financial costs; patients are admitted to hospital for costly neuroimaging as well as other investigate tests (Marshall et al., 2012). Most costs are indirect and accumulate from computed tomography (CT) scans or emergency department visits and stays (Borg et al., 2004). In particular for pediatric brain injury, the estimate of the cost was over $1 billion dollars annually in the United States (Schneier et al., 2006).

1.1.2 Pathophysiology following brain injury

The cause of these long term behavioural deficits is complex and related both to the nature of the primary event as well as the secondary cascade that follows the injury and possibly invoking further cellular damage or conversely restoration. In the case of a focal external hit, there is typically primary damage after the initial force of the injury, when the force is transmitted though the skull and when the brain is exposed to mechanical tissue deformation (Zhang et al., 2004). The site of the injury is termed the coup and the impact usually results in the brain being pushed against the opposite side of the skull, which is known as the contrecoup location (Zhang et al., 2004). However, depending on the type of hit and location, the brain may also be exposed to angular acceleration, compression, shearing, and torsion of blood vessels, neurons, glia and axons (Ommaya et al., 2002; Xiong et al., 2013). Primarily in mTBI the mechanical forces may initially cause cellular or axonal injury but most of the damage is believed to recover over time and there is typically no overt structural damage as seen by imaging (Iverson, 2005; McCrory et al., 2009). Depending on the force of the impact there may
be swelling of brain tissue and increased intracranial pressure (ICP) and in more severe cases disruption of the blood brain barrier (BBB) (Başkaya et al., 1997; Lenzlinger et al., 2001).

In addition to the primary injury, there is a secondary injury response that is primarily caused by the release of excitatory amino acids such as glutamate and aspartate, which can induce a cytotoxic influx of Ca\(^{2+}\) (Xiong et al., 2013). The high levels of Ca\(^{2+}\) create a neurotoxic environment which results in an increased activation of the Na\(^+\)/K\(^+\) pump and an overall increased metabolic demand (Giza and Hovda, 2001). This can lead to mitochondrial dysfunction, oxidative damage from an increase in free radicals, and impaired microtubule formation within axons, specifically in the white matter tracts (Giza and Hovda, 2001). In some rodent models, there has been an increase of 46% glucose metabolism after mTBI (Barkhoudarian et al., 2011). An increase in the metabolism of cells combined with a slight decrease in cerebral blood flow and mitochondrial dysfunction, may result in cellular distress that could contribute to functional deficits following mTBI (Giza and Hovda, 2001). There is some preliminary works showing that if rats are subjected to a ketogenic diet and deprived of glucose after mTBI the behavioural deficits may be alleviated (Appelberg et al., 2009).

In addition to mitochondrial damage, there is also a sterile inflammatory response that is initiated by the resident immune cells of the brain, known as microglia (Readnower et al., 2010). This response of inflammatory markers has been extensively studied in moderate and severe injury; but it is unclear whether the same sequelae would occur for milder injuries. Following TBI preliminary neuroinflammation and swelling occurs and peripheral lymphocytes have been shown to infiltrate the CNS in the first 24 hour period (Lenzlinger et al., 2001). The breakdown of the BBB could allow additional neutrophils, lymphocytes and other immune cells found in the
periphery to invade into the brain, move to the injured site and either mediate cell survival or apoptosis (McIntosh et al., 1998). The severity of injury influences how long the BBB takes to recover: a mouse model of TBI demonstrated micromolecular leakage for 90 minutes after injury, whereas a greater impact led to persistent leakage for up to 24 hours (Yang et al., 2013). Both primarily and secondary injuries may contribute to cellular damage and functional deficits and depending on the force of the injury it may be difficult to determine which had a defining role.

1.1.3 Microglia

In cases of damage to the central nervous system, the primary immune pathway relies on resident macrophage-like cells known as microglia. Microglia originate in the yolk-sac and migrate to the brain during development (Ginhoux et al., 2013). One of their major roles is to survey their environment for indications of harm, which they do while in a ‘ramified’ or resting state (Boche et al., 2013). Traditionally it was thought that microglia had two activated polarization phenotypes, the first state ‘M1’ was characterized by the production of pro-inflammatory cytokines and the second ‘M2’ was representative of an anti-inflammatory activated state where tissue repair could occur (Cherry et al., 2014; Hanisch, 2002; Orihuela et al., 2015). In addition to dealing with different aspects of the immune response, the two states also were associated with different metabolic conditions. In the M1 state of activation glycolysis was the primary mechanism of metabolism, with increased nitric oxide and citrulline production (Orihuela et al., 2015). The M2 state of activation centered on oxidative metabolism for tissue repair, which was a longer process compared to the relatively acute M1 activation. However,
Current evidence suggests that microglia cells have heterogeneous states of activation and are not restricted to two phenotypes (Mosser and Edwards, 2008). There are more distinct classes of activation which include: M1 representative of cytotoxic properties, the M2a state with anti-inflammatory regulation, M2b and immunoregulation, and M2c with deactivation and wound healing (Chhor et al., 2013). In addition, these phenotypes are not static and there is recent evidence to suggest that microglia cells respond to both pro and anti-inflammatory signals simultaneously (Morganti et al., 2016). Regardless of their classification, microglia are gaining recognition for the mediation of multiple neurodegenerative diseases and adult TBI (Karve et al., 2016; Xu et al., 2015). Their role in cases of paediatric and more mild injuries has been less well characterized.

1.1.4 Linking microglia and mTBI

Microglial cells are an important source of cytokines, chemokines, and trophic factors and act as part of the immune system in the brain. Microglia have been shown to play a role in mTBI, for example after damage to the central nervous system microglial cells respond to the injury and shift to an activated phenotype (Ziebell and Morganti-Kossmann, 2010). There is evidence that microglia become activated following mTBI in experimental models, when induced with a blast injury (Readnower et al., 2010). In a separate model of mTBI utilizing a controlled cortical impact, microglia were activated for up to a year after injury, this coincided with lesion expansion, white matter damage and damage to hippocampal cells (Karve et al., 2016). As further evidence that microglia activation can be chronic, one study has reported that after mTBI microglia were sensitized for a month following injury, essentially microglia cells
responded with an exacerbated cytokine production in the presence of an additional challenge (Muccigrosso et al., 2016). In addition to findings in experimental models there is some clinical evidence, that this activation of microglia is long-term as positron emission tomography (PET) scans revealed activation up to 11 months for patients with moderate to severe brain injury (Ramlackhansingh et al., 2011). Although in most cases the clinical symptoms of mTBI resolve, the chronic sensitization of microglia cells may put individuals at risk for greater response in repeat injuries.

1.1.5 Animal Models of TBI

Animal models have been widely used to better understand the pathophysiology following injury and attempt to create effective treatment options. The two most common animal models of TBI used are the fluid percussion injury (FPI) and controlled cortical impact (CCI). The FPI relies on the animal being anesthetized, a section of the skull-cap being removed to expose the brain and then a fluid pressure pulse striking the brain laterally (Thompson et al., 2005). This model creates intracranial haemorrhage, brain swelling resulting from increased ICP and replicates some clinical features of TBI (Shultz et al., 2012; Xiong et al., 2013) The CCI model similarly requires the animal head to be fixed in position and the skull to be removed (Shohami et al., 1997; Xiong et al., 2013). However, this model relies on a piston to impact the brain with a controlled speed and velocity (Xiong et al., 2013). Between different models there are differences in both symptomology and molecular profiles, for example there are changes in brain derived neurotrophic factor (BDNF) for the FPI model at 3, 6, and 24 hours after injury in the hippocampus but only changes for CCI at 12 hours in the cortex (Marciano et al., 2002). The
main advantage for both the FPI and CCI models is that they induce reproducible and reliable hits and the devices can produce the same force of injury. However, in both of these models the rodent is placed under anesthetic for an extended period and undergoes surgery which can increases the mortality rate (Xiong et al., 2013). Therefore, for this study a modified controlled weight-drop which utilized light anesthetic and a secondary fall was employed to create a mild injury that is most similar to a sport-related concussion (Mychasiuk et al., 2014a).

1.1.6 Prevention, Treatment and Biomarkers

Currently one of the most important issues with the treatment and diagnosis of mTBI, is that there is no reliable way of determining which people will recover quickly (‘resilient’) or those that will have detrimental long-lasting symptoms (‘risk’). Previously, a wide range of proteins that are markers of an injury have been examined as biomarkers for prognosis such as S100B (a Ca^{2+} binding protein primarily found in astrocytes), glial fibrillary acidic protein (GFAP) which is released by astrocytes after mTBI, and myelin basic protein (MPP) that is expressed by oligodendrocytes and has been shown to accumulate after damage to axons (Jeter et al., 2012). However, although these proteins may indicate the presence and severity of TBI, a person’s outcome following injury remains unclear. In addition, although a mTBI may be identified there are few effective treatments for improving outcomes (Comper et al., 2005). The typical course of treatment relies on a break from activity but the amount of rest time recommended varies (Comper et al., 2005). Individuals are recommended to rest at least 7 – 10 days following sports related mTBI and the resolution of symptoms (Purcell, 2009). Persistent
individual symptoms that are present several months after injury are treated with medication or cognitive rehabilitation therapy (Rabinowitz and Levin, 2014).

Some treatment strategies have focused on combating the acute effects after injury. For example, anti-inflammatory drugs have been given to try and prevent inflammation after mTBI, which showed positive results when used in a rodent model but was unsuccessful in human trials (Maas et al., 2006; Shohami et al., 1997). In addition to anti-inflammatory drugs, other treatments such as supplementing dietary omega-3 fatty acids to help reduce oxidative stress have been tried (Barrett et al., 2014). Lastly, some treatment has focused on therapies such as cognitive rehabilitation or vestibular rehabilitation to improve long-lasting symptoms (Alsalaheen et al., 2010; Kirkwood et al., 2006).

However, treatments and therapies would be most appropriately directed if they were given to individuals who were at high risk of developing PCS. Part of the goal of this thesis was to examine a pre-existing condition that could influence which individuals are at higher risk for a poor prognosis. Identification of a sub-population at risk could help reduce costs, target resources, and improve patient outcomes.

1.2 Immune Programming from Neonatal Immune Challenges

1.2.1 Peripheral Immune Response

Similar to studies showing that early life stress can alter outcomes after mTBI, activation of the immune system after an early life immune challenge could contribute to risk or resilience. In a broad sense, the purpose of the immune system is to provide a defense against invading microorganisms and facilitate tissue repair after injury (Watkins et al., 1995a). It can be divided
into the non-specific (innate) and specific (acquired) immune response. In the initial immune response, pathogens bind to Toll like receptors (TLR) on macrophages and other immune cells which initiate the release of prostaglandins and transcription factors (Nguyen et al., 2002). Prostaglandins are capable of directly crossing the BBB and interacting with the hypothalamus. However, in addition to the immediate production of prostaglandins there is a secondary production of cytokines. TLR binding can activate transcription factor NF-κB (among other intracellular downstream mediators) which initiates the production of proinflammatory cytokines (Nguyen et al., 2002). If the levels of cytokines are sufficiently high the liver activates and alters enzymes within the blood plasma to try to prevent bacterial replication (Nguyen et al., 2002).

The immune response has been shown to have both beneficial and deleterious effects as demonstrated by the fever response (Harden et al., 2015). The immune system utilizes both a T\textsubscript{H}1 response (T helper cells 1) primarily characterized by an increase in pro-inflammatory cytokines and a T\textsubscript{H}2 response primarily characterized by the release of anti-inflammatory cytokines (Nguyen et al., 2002). The two responses act as negative feedback loops and it is thought that both are required for a healthy response to injury or inflammation (Estes and McAllister, 2014; Lenzlinger et al., 2001).

1.2.2 Cytokine communication with the brain

The immune system utilizes messenger molecules called cytokines, which are fairly large lipophobic proteins. Cytokines are not capable of crossing the BBB directly (Watkins et al., 1995a), however there are several mechanisms that allow them to affect the CNS. For example, cytokines may cross at circumventricular sites which are areas that contain excessive vasculature
without the protection of the BBB; in addition they may also bind to endothelial receptors or activate peripheral afferents (Quan and Banks, 2007; Watkins et al., 1995b). When cytokines are produced in the periphery they can either bind to the paraganglia of the vagus nerve to signal the CNS or they may be carried in the blood to interact with receptors in the endothelium or circumventricular sites. Overall, there are multiple pathways by which a peripheral insult can cause changes in the production of cytokines within the brain (Quan and Banks, 2007). Once the cytokines signal the brain they may activate cyclooxygenase-2 (COX-2) which leads to the production of prostaglandin E2 (PGE2) and activation of the hypothalamic-pituitary-adrenal (HPA) axis along with the production of fever and sickness behaviour (Dantzer and Kelley, 2007). Sickness behaviour is characterized by lack of energy or appetite, and social withdrawal, which has been well characterized in both animal and human models (Dantzer and Kelley, 2007). The immune system and the HPA axis are closely tied, which means that the immune response is susceptible to alterations from stress and vice versa.

1.2.3 *Lipopolysaccharide*

One of the most common ways of creating an experimental immune challenge in rats is to stimulate the system with an injection of lipopolysaccharide (LPS). LPS is a molecule derived from gram-negative bacterium, which is used to mimic a bacterial inflammation. When injected in the periphery it can induce a pro-inflammatory cytokine cascade by activating TLR4s which lead to the downstream production of IL-1β, IL-6 and TNF-α (Skelly et al., 2013; Turrin et al., 2001). In addition to the production of cytokines in the periphery, cytokines are independently synthesized in microglial and other cells in the brain (namely the cerebral cortex and
hippocampus) during the innate immune response (Galic et al., 2008; Turrin et al., 2001). This is traditionally associated with the microglial cells entering an M1 state of activation (Orihuela et al., 2015). Although LPS administration of 100µg/kg in a critical neonatal period has been shown to produce a short term fever response lasting 6-8 hours (Heida et al., 2004), there is also research to indicate that there are long-term changes in the brain – notably increased seizure susceptibility (Galic et al., 2008) and cellular death following ischemic stroke (Spencer et al., 2007).

1.2.4 Immune programming during critical development

If an LPS immune challenge occurs during a critical period of development, it may cause long-lasting changes (Spencer et al., 2006). Rats that are injected with LPS (100µg/kg) on postnatal day 14 have an increased production of N-methyl-D-aspartate (NMDA) receptors in the hippocampus when measured as adults (Harré et al., 2008). As NMDA receptors are crucial in the processes of long term potentiation this can affect learning and memory processes. Other work has shown that rats injected with LPS at P14 have a decreased fever response (characterized by decreased COX-2 in the hypothalamus) when re-injected in adulthood (Spencer et al., 2011). The critical period for febrile response to LPS was determined to be between P14 and P21 (Spencer et al., 2006) and this critical window could be due to the rapid changes in neurotransmitter receptor types: for example, Gamma-aminobutyric acid (GABA) switches from being an excitatory to inhibitory neurotransmitter during this period (Swann et al., 1989). It is thought that as the brain develops, the central production of cytokines associated with LPS administration, alters the brain to make it hyper excitable as an adult. This has been
demonstrated in work that has shown that rats injected with LPS on P7 or P14 have a reduced seizure threshold when tested as adults (P60) (Galic et al., 2008). Most notably for this project, one experiment has found that there is greater neuronal loss in the amygdala after ischemia in adulthood for rats that received neonatal LPS (Spencer et al., 2007). This result could have applications to mTBI in that similar to ischemia, cellular death and inflammation occur after brain injury.

1.2.5 Neonatal LPS Exposure on Behaviour

Although LPS has been shown to alter excitability in the hippocampus, and may alter selective receptors, there is little consistent evidence to show chronic behavioural deficits when exposed to LPS during the critical window. There are well-characterized acute symptoms and sickness behaviour when LPS is given, such as decreased exploration in an open field setting, an inability to recognize novel objects, and increased pain-sensitivity to hot-plate or tail-flick tests (Bossù et al., 2012; Yirmiya et al., 1994). The short-term effects of an illness on behaviour have been well characterized as sickness induced behaviour and may include fever, hypersensitivity to pain, and reduced motivational drive (Dantzer and Kelley, 2007). These acute behavioural changes can be adaptively beneficial as they allow the animal to withdrawal and rest, in an effort to save energy to pay for a costly immune response.

Tests performed on rats injected with LPS during early life between P7 and P14 have found that there were no observable changes in anxiety as measured by the elevated plus maze (EPM) at approximately P80 (Spencer et al., 2006). Research examining the behaviour of rodents exposed to LPS on anxiety-tasks is quite variable, and depends on the time of the exposure,
dosage, and when the animals are tested (adolescence or adult-hood) (Bilbo et al., 2008; Dinel et al., 2014; Walker et al., 2009). Despite relatively few overt differences in behaviour, there is an underlying susceptibility when LPS is given during the neonatal critical window. This vulnerability is typically exposed when animals are subjected to a second stressor or subsequent injury (Espinosa-Oliva et al., 2011). Although the behavioural deficits provoked by early exposure to LPS have not been consistent, this study will explore a more comprehensive battery of tests to determine if neonatal LPS alone, and LPS combined with mTBI, induces notable behavioural alterations when tested in greater detail. In addition, there have been few studies that have included and directly compared males and females in these responses.
1.3 Goals, Hypothesis, and Rationale

1.3.1 Goals

The goal of this project was to ask if immune programming during development would alter the outcomes of a later mTBI, in an attempt to better understand why some children are at greater risk for developing PCS.

1.3.2 Susceptibility or Resilience

It could be expected that similar to the increased cellular death following ischemia (Spencer et al., 2007), there would be an increased cellular stress response in the brain following mTBI which could result in greater cell death. In addition, there is evidence that neonatal LPS results in some brain areas, such as the hippocampus, becoming hyper-excitable. A brain region that is excitable could have an enhanced neurotoxic response since excitatory amino acids and Ca$^{2+}$ influx occurs after mTBI. There could be greater release of pro-inflammatory cytokines which would tip the scale to promote cellular death that exceeds the repair capacity. This imbalance could be associated with either a detrimentally enhanced T_{H}1 response or an increased rate of apoptosis. Under these circumstances rats that experienced both an injection of LPS and mTBI could exhibit a significantly impaired performance on the behavioural battery and an altered cytokine profile in comparison to control animals.

Conversely, there is some support that an early life illness is protective for depressive-like and anxiety-like behaviours (Bilbo and Schwarz, 2009). Rats treated on P4 with the bacteria Escherichia coli were found to be resilient to stress-induced depressive-like behaviour (Bilbo et
al., 2008). From this work, it would appear that an early life immune challenge confers some susceptibility but this occurs with selective resilience as well. Therefore when compared to SAL injected animals, the exposure to early life LPS could contribute to protective effects when the rat experiences an additional injury (mTBI) at P30, which would result in a relatively ‘normal’ or even enhanced performance on the test battery.

1.3.3 Hypothesis

As discussed above, the experimental question of this study was whether neonatal LPS exposure modified outcomes after mTBI and could therefore be part of the growing body of evidence validating the relevance of pre-existing factor on mTBI outcomes. Thus the hypothesis of the study is that a neonatal (P10) LPS exposure affects both the behavioural outcome and inflammatory molecular markers examined following mTBI at P30.

1.3.4 Approach

Sprague Dawley rats were injected intraperitoneally with LPS or SAL at P10 and then given mTBI or Sham injury at P30, as shown in Figure 1. One group of animals were sacrificed 24 hours after injury to examine acute effects; the second larger group went through rigorous behavioural testing and then were sacrificed at P45. At sacrifice brain tissue from the hypothalamus, prefrontal cortex, dorsal and ventral hippocampus was collected and evaluated for differences in mRNA levels of the selected cytokines. Trunk blood was also collected and tested
for changes in peripheral cytokine levels to examine both acute and chronic molecular changes resulting from mTBI and LPS.

**Figure 1. Schematic representing the experimental design: Testing the effects of neonatal LPS (P10) and mTBI (P30) on behavioural measures and ‘acute’ and ‘chronic’ molecular changes.**

1.4 Model and Behaviour Rationale

To model an acute inflammatory event, a 100µg/kg dose of LPS was administered at P10 which is within the critical window shown to be capable of inducing persistent changes in subsequent immune activation and brain excitability. In addition P10 is a key period for the GABAergic system, as it is undergoing changes in activity (Swann et al., 1989). An exposure to LPS during this window has been shown to increase GABA_A α5 receptors and mRNA expression of Na-K-Cl cotransporter (NKCC1), both of which contribute to an excitatory brain state (Reid et al., 2013). The injury model chosen in this study was a weight-drop model that has been
extensively validated in terms of symptomology similar to PCS (Mychasiuk et al., 2014a). One of the reasons this model was chosen is because it creates a heterogeneous injury similar to clinical presentation. In addition, it more closely resembles a sports related mTBI (with dynamic forces and a secondary impact) when compared to the more traditional fluid percussion injury (FPI) and controlled cortical impact (CCI) models which utilize a fixed head. The behavioural battery was chosen to assess measures of motor dysfunction, anxiety, social interactions, memory, and depressive-like behaviour. As multiple behaviours are tested, if deficits are seen in tests that evaluate the same criteria (open field and elevated plus maze are both used to test anxiety) then this provides stronger evidence than a single behavioural test. While there may be concerns that previous tests alter subsequent behaviour, there is evidence to suggest that the majority of behaviours show few differences whether done singly or as part of a battery of tests (Lad et al., 2010).

1.5 Brain Region and Cytokine Rationale

1.5.1 Selected Brain Regions

The hypothalamus (HYPO) was chosen as a region of interest because it plays a central role in the immune and stress response (Beishuizen and Thijs, 2003; Chrousos, 1995). The hypothalamus acts as a regulator of the hypothalamic-pituitary-adrenal (HPA) axis. Briefly, when animals experience stress corticotropin-releasing hormone (CRH) is released primarily from the paraventricular nucleus within the hypothalamus and activates areas in the anterior pituitary which cause the release of adrenocorticotropic hormone (ACTH). When ACTH is identified by peripheral receptors in the adrenal glands it triggers the production of epinephrine,
norepinephrine, and cortisol (Charmandari et al., 2005). Glucocorticoids and other products of the stress response act to negatively regulate the immune system and the active production of cytokines (Charmandari et al., 2005). Therefore, as the experimental animals in this study were subjected to an immune challenge, sterile brain injury, and mild stressors the hypothalamus was a key area of interest.

The prefrontal cortex (PFC) plays a key role in executive functioning and top-down decision making for behaviour that requires intent to implement (Miller and Cohen, 2001). This region was chosen for two important reasons; firstly, this injury model uses a 100 g weight which drops over the midline of the skull, including the prefrontal cortex and is therefore a primary site of injury (Mychasiuk et al., 2014a) and secondly mTBI patients often report alterations in anxiety, memory, and depressive-like behaviour (Barlow et al., 2010) which are thought to be mediated in part by the PFC. These behaviours are tested in this design through the open field, elevated plus maze, and forced swim tasks, which have been shown to be sensitive to PFC modulation (McKlveen et al., 2013; Shah and Treit, 2003). As the PFC is an important region for several behaviours examined, it was also important to try and find both acute (24 hours) and long-term cytokine response in this region. Although not causational evidence, a difference in cytokine mRNA coinciding with a change in behaviour links the inflammatory state of the animal and the behavioural phenotype.

Lastly, the ventral and dorsal hippocampus (VHPC and DHPC) are collectively responsible for many aspects of memory including spatial and contextual (Jarrard, 1993), as well as other integrative functions. The hippocampus can be anatomically and functionally divided based on contributions to different aspects of memory such as: spatial organization and
orientation in the dorsal hippocampus and more emotional aspects in the ventral hippocampus (Fanselow and Dong, 2010). In addition, previous work has found that immune challenges may selectively alter select genes in the hippocampus such as NMDA receptors (Harré et al., 2008) or impart increased susceptibility to seizures (Galic et al., 2008). As LPS might selectively cause molecular changes within the hippocampus, this is also an important area to examine. Overall the DHPC and VHPC are important for both behavioural tests and also are regions more susceptible to alterations from the neonatal exposure to LPS.

1.5.2 Molecular Markers

The focus of this study was the role of immune programming on neuroinflammation following mTBI. In previous work, mTBI markers studied have included TNF-α and IL-6 (Shohami et al., 1994), with some studies examining IL-1β, IL-8, IL-10, or TGF-β (Cederberg and Siesjö, 2010). This overlaps with the markers shown to be increased after LPS injection, most specifically IL-1β and TNF-α (Skelly et al., 2013; Turrin et al., 2001). As TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines and the balance between pro and anti-inflammatory is critical in the immune system, the predominant anti-inflammatory cytokine IL-10 was also included in this study. After initial interpretation of the cytokine mRNA results two indicators (Arg1 and iNOS) of microglia/macrophage phenotype were included, as microglia activation has been shown to regulate the response to LPS and mTBI (Espinosa-Oliva et al., 2011; Ramlaclkhansingh et al., 2011). The four cytokines examined are discussed below, with literature examining their role in either mTBI or LPS exposure.
1.5.3 Tumor Necrosis factor alpha (TNF-α)

TNF-α plays a role in multiple pathways: the NF-κB signal pathway as an immune mediator, the mitogen-activated protein kinases (MAPK) pathway as primarily an activator of cell differentiation, and finally the apoptosis pathway (Saklatvala et al., 2003). Although a complex signalling molecule, its role will be discussed as it pertains to inflammation. TNF-α is a pro-inflammatory cytokine, which is synthesized by multiple types of immune cells in the periphery, but primarily from microglia and macrophages within the brain (Hanisch, 2002). TNF-α is one of the first cytokines produced after an immune challenge or injury and has a main role in regulating the cytokine cascade (Ziebell and Morganti-Kossmann, 2010). It has two receptors: TNFR1 and TNFR2, which are important in apoptosis and neuroprotection respectively (Lenzlinger et al., 2001). TNFR1 is expressed on most cell types and mainly results in the activation of transcription factors NF-κB and Activator protein-1 (AP-1) (Probert, 2015). TNFR2 is conversely found in few cell types and is mostly limited to T immune cells, endothelial and oligodendrocytes, where is primarily acts to induce cell proliferation (Probert, 2015). TNF-α activates a similar pathway to IL-1α or IL-1β and together the cytokines are thought to mediate inflammation following trauma.

TNF-α expression after mTBI has been characterized both in clinical and experimental models; there is elevated in the CSF of human patients and elevated mRNA and protein levels in rodent brains following moderate or severe TBI (Lenzlinger et al., 2001). However, evidence is conflicting about whether the levels of TNF-α are beneficial or detrimental following mild or moderate TBI. One study used dexanabinol, a synthetic cannabinoid and an NMDA receptor antagonist, after experimental closed head injury. The antagonist inhibited TNF-α production and
the rats had better motor function and BBB integrity compared to controls (Shohami et al., 1997). However, when used in a clinical trial of severe TBI, dexanabinol showed no benefit in outcome (Maas et al., 2006). In addition, TNF-α KO mice showed an increased amount of tissue and BBB damage, and a poor recovery, following brain injury (Scherbel et al., 1999). Therefore, TNF-α is believed to mediate the outcomes after TBI but it appears to have dual roles, involved in both protection and damage, depending on both the pathway of activation and the level of the response. With respect to peripheral immune challenges, the injection of the same dose of LPS (100 μg/kg) used in this study has been shown to increase pro-inflammatory cytokines in the brain, with both TNF-α and IL-1β showing upregulated mRNA expression in the cortex, hippocampus, and cerebellum after injection (Skelly et al., 2013; Turrin et al., 2001).

1.5.4 Interleukin-1β (IL-1β)

Similar to TNF-α, the IL-1 family of cytokines has been shown to be an initiator of the immune response and the production of a cytokine cascade (Ziebell and Morganti-Kossmann, 2010). Although there is more recent evidence that some cytokines in the IL-1 family have a role in mRNA splicing (Luheshi et al., 2009). Within the IL-1 family the two most commonly studies cytokines are IL-1α and IL-1β, however in the context of TBI IL-1β is better characterized. It has been shown to play roles in the production of prostaglandins, activate COX-2, BBB disruption, and apoptosis (Woodcock and Morganti-Kossmann, 2013). Specifically, studies have found elevated levels of IL-1β in the CSF of both rodents and humans following more severe TBI injuries (Woodroofe et al., 1991). Additionally, damage following TBI or ischemia was increased when IL-1 receptor A (acting as an antagonist for the receptor) was co-injected (Relton
and Rothwell, 1992). Similar to experimental results for TNF-α, there has been some success when using a pharmacological intervention. One study administered minocycline, an antibiotic that reduces caspase-1 (an important enzyme in converting IL-1β into its mature form), and mice demonstrated reduced lesion volumes after TBI (Sanchez Mejia et al., 2001). In addition to the work done in mTBI there is evidence that IL-1β is produced when animals experience peripheral immune challenges. The intraperitoneal injection of LPS is associated with increased IL-1β mRNA expression in the cerebellum, cortex, hippocampus, liver, spleen, and adipose tissue (Skelly et al., 2013; Turrin et al., 2001). High levels of IL-1β are thought to play roles in both sickness behaviour and HPA axis activation (Charmandari et al., 2005; Dantzer and Kelley, 2007).

1.5.5 Interleukin-6 (IL-6)

Secreted by macrophages, astrocytes, and neurons IL-6 has multiple functions, and is capable of aiding in both apoptosis and wound repair (Ziebell and Morganti-Kossmann, 2010). It may also act to negatively regulate IL-1β and to directly or indirectly induce neurotrophic factor expression such as nerve growth factor (NGF), therefore potentially promoting neural recovery (Lenzlinger et al., 2001; März et al., 1999; Sharma and Laskowitz, 2012). As for its role in mTBI, IL-6 has also been shown to increase acutely 8 hours after injury in one model of TBI that utilized a closed head impact injury (Shohami et al., 1994). Another study with IL-6 KO mice found greater levels of tissue damage after injury, indicating that IL-6 could be neuroprotective (Penkowa et al., 2000). In addition to being involved in mTBI, previous work has shown that IL-6 is a key mediator in the fever response and COX-2 production (another hallmark feature of
sickness behaviour) during peripheral immune activation (Eskilsson et al., 2014). IL-6 is involved with prostaglandin synthesis, and acts by binding to endothelial receptors in the vasculature of the CNS. Similar to TNF-α and IL-1β, there was an elevation of IL-6 after exposure to LPS, in this case the peripheral levels of IL-6 was elevated 2 hours after exposure (Skelly et al., 2013). Similarly, brain mRNA was increased in both the hippocampus and hypothalamus 2 hours after LPS exposure (Skelly et al., 2013).

1.5.6 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that is produced by both microglia and astrocytes in the CNS, and macrophages in the periphery. It acts to suppress microglia and astrocyte activation as part of a negative feedback loop, while also decreasing the production of pro-inflammatory cytokines. IL-10 is produced by microglia cells in an M2c state of activation that aids in repairing damage after injury (Chhor et al., 2013; Orihuela et al., 2015). In rodents IL-10 seems to be increased during recovery from mTBI, but human literature regarding children with severe mTBI indicates that those with high levels of IL-10 in their CSF were at a greater risk for a poor outcome (Bell et al., 1997). It is possible that a poor outcome response is associated with IL-10’s ability to lower TNF-α production, which in turn reduces the positive effects of the pro-inflammatory response. The effects of using anti-inflammatory compounds in a clinical population is mixed (Bell et al., 1997; Patterson and Holahan, 2012) with some studies showing selective benefit, no changes, or detrimental effects. From the combined literature examining cytokines and immune response after injury it appears that there is variation in both the time of the response and model used. It also appears that both a pro and anti-inflammatory response of
cytokines is important to successfully recover from injury or illness (Harden et al., 2015; Lenzlinger et al., 2001).
1.6 Experimental Methodology

1.6.1 Major goals

The first aim of this project was to determine whether or not neonatal exposure to 100µg/kg of LPS on P10 significantly altered behavioural or molecular markers at a later age. The second was to determine the effect of mTBI alone on behavioural and molecular markers using an animal model extensively validated in our lab (Mychasiuk et al., 2014a). Following injury a behavioural test battery was used to detect motor, cognitive and social impairments consistent with those seen in poor outcomes after clinical mTBI. The third and primary aim however, was to determine if neonatal exposure to LPS altered outcomes after mTBI. All three aims were evaluated acutely at P31 and chronically after extensive behavioural testing at P45.

1.6.2 Injections and Housing

Twelve dams were bred in-house to avoid transport stress (Moriyama et al., 2013) and housed in a vivarium that was maintained on a 12:12 hour light and dark cycle (7am – 7pm), where the animals had access to food and water ad libitum. Both mothers and pups were handled daily to familiarize them with the experimenter. At P10 pups were briefly separated from their mothers and subjected to intraperitoneal (i.p.) injection with sterile solutions of either 100µg/kg LPS (Escherichia coli: 026:B6) or an equal volume of saline (SAL) solution. Rats with LPS had their tails coloured for the next eleven days until weaning (P21), when ear notches were used for identification. Rats were monitored for changes in body weight over the 48 hours post-injection. At P21, rats were separated from their mothers and housed in sex-matched cages of four, with two LPS and two SAL animals in each cage. A trial group consisting of 22 animals (from two
dams) was run in November of 2014; a larger group consisting of 80 animals (from ten dams) was run in December of 2014. As there were no differences between these two groups, the data was pooled. Care was taken to ensure that each individual experimental group contained animals from several litters to ensure that litter effects were minimal.

1.6.3 mTBI

At P30, rats were subjected to either a closed head mTBI using the modified weight drop technique or Sham injury. Rats were briefly anesthetized with isofluorane and then placed on a scored piece of tinfoil suspended over a sponge cushion. A weight (150 g) was released by the experimenter and dropped from a height of 0.5 m to impact the midline of the head, just posterior to the Bregma suture. After the impact the rat underwent a 180° rotation and fell onto the cushion, which exposed the brain to a secondary impact and associated rotational forces. After the injury, lidocaine was applied topically to the site of the injury and the rat was placed in a supine position to recover. The time-to-right was measured from the time of the impact to the time that the rat assumed a prone position. Sham animals were also lightly anesthetized and placed under the same apparatus but did not experience the weight drop injury.

1.6.4 Behavioural testing and scoring

All the following behavioural tests were conducted in the light portion of the animal’s circadian cycle (between 7 am – 7 pm). The behaviour was scored by a blinded observer who was unaware of the animal’s injury and exposure to LPS or SAL. The open field was not scored
by an observer, but instead utilized computer tracking software (Ethovision XT). Between trials, the apparatus was cleaned with Vircon®. The order of the tests was chosen to minimize the interference one test would have on the subsequent tests (tests were done from least to most stressful). In addition, it has been suggested that there is no difference in performance when animals undergo a single test or multiple tests performed as part of a battery (Lad et al., 2010).

1.6.5 Beam Walking (P31)

The beam walking test was used to measure motor dysfunction. At P31, approximately 24 hours after injury, rats were placed on a long tapered beam consistent with previous methodology described by Schallert. The beam had safety edges on either side (2cm wide) that provide support should the rat slip from the main beam. For the first trial the rat was prompted to walk from the start of the beam to its home cage and upon arrival given a reinforcement interval of sixty seconds as a positive reward. The next four trials of the rat crossing the beam were videotaped. The reward interval given at the home cage was given at the end of each trial. The behaviour was scored by measuring the number of hind legs slips and the time-to-cross for each interval. Rats that fell off the beam during a trial were scored as having performed three foot slips, given the severity of the motor dysfunction.

1.6.6 Open Field (P32)

The open field was used to measure anxiety, as animals will demonstrate a preference for the periphery or center of the open field (Blanchard and Blanchard, 1988). Animals were tested for 10 minutes at P32 in an open circular field (diameter 135 cm, height 45cm) under normal
lighting conditions (560 lux) similar to (Mychasiuk et al., 2014a). An overhead camera with tracking abilities measured the animals’ total movement and time spent in the outer and inner portions of the circle (Ethovision XT). The amount of time spent in the inner portion was deemed the ‘exposed time’.

1.6.7 Elevated Plus Maze (P33)

The elevated plus maze was also used to test anxiety, measured by the relative amount of time a rat spends in the closed or open arms of the maze (Walf and Frye, 2007). On P33, rats were placed in the center of an elevated plus maze approximately 55 cm above the ground. The maze consisted of two open and two closed arms connected by a center portion. A video camera was placed so that both open arms and the center of the maze could be clearly seen for the 5 minute trial. An experimenter scored the data for how much time the rat spent in the open, closed and center arms of the maze.

1.6.8 Novel Context Mismatch (P35 – 38)

The novel context mismatch relied on the animal’s ability to distinguish a novel and a familiar object within a contextual environment; this was used as a measure of working memory. For three consecutive days (P35-37), rats were trained in two different condition cued environments similar to that described by (Spanswick and Sutherland, 2010). Context A was a clear rectangular box (70 x 40 x 33 cm) equipped with two rectangular sample trays and Context B was a dark oval box (47 cm diameter, 36 cm high) equipped with two cylindrical cola cans. The rat was placed for 5 minute intervals in Context A moved to Context B for five minutes and
then returned to the home cage. On the probe day, P38 the rat was placed in Context A, Context B and then given a 5 minute delay in the home cage before being placed into Context C. Context C was a dark square box with one object from Context A and one object from Context B. A video camera was stationed to enable observation of the rat for the five minute probe trial. The video was scored for the amount of time the rat spend exploring both objects. The object that didn’t belong in the context (from Context A) was called the ‘novel’ object and the total amount of time was defined as the ‘exploratory time’. The amount of time spent with the novel object as a fraction of the exploratory time was calculated.

1.6.9 Play (P41 – P42)

The play paradigm was used to measure social interactions with cage mates. On P41 rats were isolated for 24 hours to maximize the amount of time spent interacting. On P42 rats were placed into a clear rectangular box in previously determined pairings. The lights were shut off and the rats were allowed to play for 10 minute intervals, while an infrared camera recorded the session. Ultrasonic vocalizations were also recorded during the play session. The play behaviour was scored for complete rotations, partial rotations, horizontal rotations, attacks, non-responses, and escapes as described by (Pellis et al., 1997). Behaviour was scored according to the following definitions of social behaviour. Attacks: The ‘attacking’ rat seeks to initiate play by mouthing the back of the others neck. Complete rotations: The rat that is attacked rotates into a supine position on its back. Partial rotations: The rat that is attacked responds by moving onto a side or is partially pinned. Horizontal rotations: The rats assume a stance on their hind legs and
use their fore paws predominantly. Non-responses: After an attack is made, the rat makes no discernable response. Escapes: After an attack the rat moves or jumps away from the other.

1.6.10 Forced Swim (P44)

The time in which animals are immobile in an ‘inescapable’ situation, shows improvement when treated with antidepressants and is thought to be a measure of depressive-like behaviour (Detke et al., 1995). On P44 rat was moved from its home cage and placed into a clear cylindrical tank of warm water (diameter 30 cm, height 60cm). The rat was videotaped for a 7 minute trial and then dried off and returned to its cage. The video was scored for the time each rat spent immobile (defined as a cessation of active swimming motions).

1.6.11 Sacrifice (P45)

A small group of animals (n=32: 16 males and 16 females) were sacrificed 24 hours after mTBI or Sham injury on P31. The group of rats that went through behavioural testing (n=70) were sacrificed at P45. Rats were briefly anesthetised using isofluorane until unresponsive to toe-pinches. They were quickly decapitated and trunk blood was collected for ELISA analysis in Serum Separator Tubes (BD Vacutainer). Brains were removed and weighed and the hypothalamus (HYPO), prefrontal cortex (PFC), dorsal (DHPC) and ventral hippocampus (VHPC) were dissected out over ice according to the regions shown by (Zilles, 1985). Brain tissues were snap frozen on dry ice and then stored at -80°C. Serum samples were left to clot at room temperature for 30 minutes, and then centrifuged at 1000g for 10 minutes. Serum was aliquoted in 300 – 400 µl increments and also stored at -80°C.
1.6.12 **ELISA methodology**

ELISA kits were purchased from R and D systems for IL-1β, TNF-α, IL-6, and IL-10. Each kit varied slightly on the standard curve concentrations and sample size. Microplates were provided in each kit with room for 96 samples, each plate was previously coated with an antibody specific to the cytokine examined. Samples, controls, and standards were diluted onto the plate in triplicate and incubated for two hours at room temperature. After incubation, each well was aspirated and then washed five times to ensure no carry-over of liquid. The conjugate antibody to the appropriate cytokine was pipetted into the wells and incubated for two hours. The plate was aspirated and washed again and then colour reagents added to allow visualization of the quantity of protein. A BioTek Synergy H.T. plate reader and Gen5 2.00.18 software was set to 450 nm in order to read the plate. This allowed for the creation of a standard curve and calculated cytokine concentrations within the samples (pg/mL). The sensitivity of the assays ranged from 5pg/mL for IL-1β and TNF-α to 10pg/mL for IL-10 and 15pg/mL for IL-6.

1.6.13 **DNA/RNA Extraction**

Brain tissue was removed from the -80°C freezer and RNA and DNA were extracted according to the manufacturer’s directions by using Qiagen’s Allprep RNA/DNA Mini Kit. To prepare the kit, 450 µl of β-mercaptoethanol was added to RTL-Plus Buffer and the correct volumes of 95% ethanol were added to the Buffers RPE, AW1 and AW2. Two scoops of 0.5mm glass beads and 600 µl of RTL-Plus Buffer was added to each tissue sample. The samples were placed in a bullet blender and mixed on speed 6 for 5 minutes. This allowed for the tissue to be
physically and chemically homogenized by the breaking of disulfide bridges. The supernatant was collected and spun through the Allprep DNA spin column at 10 000 rpm for 1 minute. This allowed for the binding of DNA from the supernatant while the RNA passed to the flow-through. To isolate RNA, the flow-through was first mixed with 450 µl of 70% ethanol. The sample was then transferred to the Allprep RNA spin column. Both RNA and DNA columns were purified by washing with the buffers: RW1 (650 µl) and RPE (two washes of 500 µl) or AW1 (500 µl) and AW2 (500 µl). The RNA or DNA is eluted out of the columns by 30µl of RNase-free water or 50 µl of EB Buffer. Samples were read on a Nanodrop spectrophotometer (at A260/A280 ratio) to ensure both content and purity. The spectrophotometer should show values of between 1.8 and 2.2 at 260/280 absorbance and greater than 1.7 at 260/230, otherwise there may be phenol or protein contamination.

1.6.14 Quality Assessment of RNA

Samples from the hypothalamus, prefrontal cortex, and ventral hippocampus indicated they were free of genomic contamination based on the Nanodrop absorbance ratios. However, the dorsal hippocampus contained unusually high levels of genomic DNA (values of over 1000 ng/ul). Therefore, the quality of RNA was tested on an agarose gel. To construct the gel, 0.5g of agarose powder was mixed with 50 ml of 1x a buffer containing Tris base, Acetic acid and Ethylenediaminetetraacetate (TAE). The mixture was melted in the microwave until the agarose was dissolved. After cooling, 1.5 µl of ethidium bromide was added and then the agarose mixture was poured into a gel mold and left to solidify. RNA from all brain regions (hypothalamus, prefrontal cortex, dorsal and ventral hippocampus) was tested, 1 µg of 3
randomly chosen samples was diluted with the loading buffer. Samples and a control with only loading dye were added to the wells. The gel was run at 100 V for 30 minutes and then imaged on the BioRad Gel Doc Imager. Samples from the hypothalamus, prefrontal cortex, and ventral hippocampus contained bands that were twice the size at 28S compared to 18S and therefore considered free of DNA contamination. However, the dorsal hippocampus did contain DNA contamination. Dorsal hippocampal samples were ultimately not used in qPCR but otherwise they would have been purified by adding DNase I which helps removed DNA contamination in RNA samples.

1.6.15 cDNA Synthesis

To ensure that all amounts of RNA are equal for the cDNA synthesis reaction, the amount of volume needed to produce 1µg was calculated. This was taken from the Nanodrop calculation given in ng/µl and divided by 1000 to reach µg/µl. In order to calculate the volume of RNA required the inverse of µg/µl was taken. RNAse free H₂O made up the remainder of the 16µl reaction. When calculations were complete, PCR tubes (0.2mL) were taken and appropriate amounts of RNA and RNAse free H₂O added. Then 4µl of qScript XLT cDNA Supermix was added to the reaction, which contains reverse transcriptase, oligo primers, annealing buffer, and MgCl₂, to allow for one cDNA strand to be synthesized for each original mRNA strand. For replication of cDNA the tubes were then placed in a PCR machine and subjected to the following protocol: 25°C for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes and then cooling back to 4°C to allow for the reverse transcriptase to become active (37-42°C) and inactive. Once the
reaction was finished stock cDNA was at 50 ng/µl and then diluted to 10 ng/µl (working qPCR solution) both were stored at -20°C.

1.6.16 Primer Design and qPCR reaction

Primers for TNF-α, and IL-10 were taken from the published papers (Berti et al., 2002; Milligan et al., 2006) and primers for IL-1β and IL-6 were designed using Primer3 (http://bioinfo.ut.ee/primer3) with the following considerations: melting temperature, GC ratio, and product size in base pairs. Two primers for each cytokine were ordered and the optimal primer was used for testing. Primers that had not been previously published were run on a gel to ensure correct amplicon size. Each qPCR reaction consisted of samples run in duplicated with 1µl of cDNA combined with 0.625 µL of both the forward and reverse primers, 10 µL SYBR Green FastMix and diluted in nuclease-free water to a total volume of 20 µL. A standard curve consisting of pooled cDNA diluted from 50 ng to 0.78125 ng. The plate was read by a Biorad CFX Connect Real-Time PCR Detection System and the relative gene expression analyzed relative to two housekeeping genes - Ywhaz and CycA (Bonefeld et al., 2008). All plates were required to have efficiency ratings between 90 – 110% to be included in analysis. The expression of the chosen cytokines was calculated using the \(2^{-\Delta\Delta C T}\) as described by (Pfaffl, 2001). Essentially, this method of calculation calculates the average expression on the desired gene on the plate of samples and normalizes the expression of each sample to the average. Then that expression was normalized to both housekeeping genes, meaning that each value is representative of the quantity of mRNA in comparison to the housekeeping values.
1.6.17 *Statistics*

One-way or two-way ANOVAs were run using GraphPad Prism (version 4; La Jolla, CA) for neonatal exposure (LPS or SAL) and injury (mTBI or Sham) or sex (male or female) examined as factors and a $p$ value $<0.05$ was considered statistically significant. Bonferroni post-hoc tests (as indicated by t values) were conducted when applicable. On figures, sex differences are indicated with # and differences due to within-sex controls (LPS or mTBI) are indicated with *. 
1.7 *Aim One: Effects of exposure to neonatal LPS on behaviour and cytokines*

1.7.1 *Time-to-right and Beam Walking (P31)*

There was a significant difference in the time-to-right for males exposed to LPS but not for females (t=2.630, p<.05) (Figure 1A). There was no significant change in the number of foot slips across the beam between LPS and SAL Shams. However, there was an increase in the average time-to-cross, with both males and females taking significantly longer than SAL controls (F=8.552, p<.01) (Figure 2C).

![Figure 1A](image1.png)

![Figure 1B](image2.png)

![Figure 1C](image3.png)

**Figure 2. Decreased time-to-right and increased time-to-cross if rat is exposed to neonatal LPS.**

A) The time-to-right after Sham injury for rats exposed to neonatal LPS or SAL. There was a significant decrease for time-to-right for male rats exposed to LPS. B) No significant differences were found in foot slips across the beam between groups or sex. C) Both females and male LPS rats had an increased time-to-cross during beam walking compared to SAL controls. For all graphs female n=12, males n=16. (**: p<.01, *: p<.05)
1.7.2 Open Field (P32)

There were no changes in the distance travelled in the open field (Figure 2A) for either male or female animals, although females tended to move a greater distance during the trial. There was no significant difference between rats exposed to LPS or SAL for the amount of time spent in the center of the open field ($p > .05$), although there was a trend for increased time in the center for male LPS animals (Figure 2B).

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 3. Neonatal LPS does not alter open field activity.**

A) Total distance moved in the open field during a ten minute trial. B) No difference in the amount of time spent in the center of the open field. n=9 for females and n=12 for males.
1.7.3 Elevated Plus Maze (P33)

There were no significant differences in either males or females for the time spent in the closed arms when rats were exposed to neonatal LPS or SAL. Although both female and male LPS rats tended to spend less time in the open arms of the maze, the difference was not significant \((F=4.036, p=.06)\).

**Figure 4. Neonatal LPS does not alter open or closed arm time in the elevated plus maze.**

A) The amount of time the rat spends in the closed arms of the maze was not affected by LPS exposure. B) The amount of time spent in the open arms was not different between SAL and LPS groups, although there is a trend for LPS rats to spend less time in the open arms compared to SAL. \(n=9\) for females and \(n=12\) for males.
1.7.4 Novel Context Mismatch (P35-38)

To evaluate working memory, the percent of time the animal spent exploring the novel object of its total exploratory time was used. There was no difference between SAL and LPS Shams for either males or females ($p>.05$). In addition, there was no significant change in the total amount of time an animal spent exploring the objects.

Figure 5. Neonatal LPS alone did not alter working memory in the novel context mismatch task.

A) Percent of the time spent with the novel object does not change from neonatal exposure to LPS. B) Exploratory times in the novel context mismatch did not significantly change between LPS and SAL rats. n=9 for females and n=12 for males.
1.7.5 Play (P41-42)

There was no significant change in the number of attacks between the play pairings, although for both male and female rats the LPS Sham + LPS Sham pairings had the lowest number of attacks. In addition, they showed the lowest percentage of rotations (complete or partial) after an attack had been made; however neither of these results were significant (p > .05).

Figure 6. Neonatal LPS alone does not alter play behaviour.

A) Shows the number of ‘attacks’ in the play pairing. B) Percent of time the animal engaged in rotations (complete, partial, or horizontal). C) Percent of time the rats responded with avoidance behaviour (evasions or non-responses). There were four play pairings each for males and females.
1.7.6 *Immobile Time (P44)*

Female LPS Shams had a tendency to spend more time immobile but this was not significant \((p>.05)\). There were no significant changes between male SAL and LPS Sham animals in the amount of time spent immobile during the task.

![Bar chart showing immobile time for females and males](chart.png)

**Figure 7.** Neonatal LPS did not modify affect measures in the forced swim test.

There was no significant change in the immobile time between SAL and LPS exposed rats. \(n=9\) for females and \(n=12\) for males.
1.7.7 Brain and Body Weight (P31 and P45)

The only significant difference in terms of brain and body weight was attributed to a sex difference with males having heavier brains and bodies at both the P31 (F=5.153, \(p<.05\) and F=7.219, \(p<.05\) respectively) and P45 (F=6.715, \(p<.05\), and F=6.289, \(p<.05\) respectively) sacrifice time points. There was no effect on brain or body weight from neonatal LPS.

![Graphs showing brain and body weight comparisons between males and females at P31 and P45.]

**Figure 8.** Males had heavier brains and body weight at P31 and P45 compared to females.

A) Brain weight for animals sacrificed on P31, showing that males had heavier brains compared to female rats. B) Male rats had a higher body weight at P31 compared to females. N= 4 for both females and males. C) Males had heavier brain weights on P45 compared to females. D) Male rats had heavier body weights on P45 compared to females. n=9 for females and n=12 for males. (\#: \(p<.05\)).
1.8 Discussion of Behavioural Changes after LPS exposure

1.8.1 LPS exposure predominantly does not alter behavioural profile

The behavioural battery of tests showed that there were only affects from the exposure to neonatal LPS on the time-to-right after Sham injury and the average time-to-cross the beam. The remaining tests of anxiety, social, memory, and affect changes showed no changes. This result supports previous work done in the lab that suggests that LPS may confer susceptibility but it is primarily only shown when the animal suffers an additional insult (Bilbo and Schwarz, 2009). For example, exposure to LPS alone did not cause deficits in elevated plus maze or sucrose preference (Kentner et al., 2010; Spencer et al., 2006), but other studies have demonstrated poor spatial memory performance or increased anxiety following a ‘double hit’ (Dinel et al., 2014; Walker et al., 2009). Previous work in this lab has shown reduced seizure threshold or greater cellular death following stroke (secondary injury) after neonatal exposure to LPS, but a secondary injury was not present in these animals (Galic et al., 2008; Spencer et al., 2007). Indirectly these results support the conclusion that the Sham injury and brief exposure to anesthetic are not particularly damaging or at least are insufficient to unmask the programming effect potentially induced by the neonatal LPS.

The only two behaviours that did show an alteration to LPS were immediately following the Sham injury and exposure to anesthetic. Neonatal LPS is known to activate NF-κB which besides being an immune regulator is also known to play a role in energy homeostasis (Tornatore et al., 2012). The effects of anesthetic have been characterized to act through the mitochondrial system (Zhang et al., 2012) and therefore it is possible that the decrease in time-to-right is a result of the two systems (mitochondrial and anesthetic) interacting. In addition, the increase in
average-time-to-cross could be indicative of a greater anxiety state but upon examining the video trials it appears that LPS rats require more prompting to reach their home cage. This could be due to the fact that they sniff around the beam and change direction more than SAL rats. There is some evidence that rats that are exposed to neonatal LPS show heightened exploratory behaviour during adolescence (Rico et al., 2010). A possible increase in exploration is supported by data showing that LPS animals tended to spend more time in the center of the open field and more time exploring in the novel context mismatch, although neither of these changes was significant. Overall, these findings suggest that neonatal exposure to LPS does not ubiquitously change behavioural phenotypes from P31 – P45 and is relatively benign.

1.9 Cytokine mRNA gene expression changes

Previous studies have shown acute changes in both pro and anti-inflammatory cytokines several hours after exposure to LPS (Skelly et al., 2013). In addition, cytokine production in both the brain and periphery typically decreased back to baseline levels by 24 hours after exposure (Erickson and Banks, 2011). However, this study was unique in several aspects: the first is that both sexes were considered while some previous studies have focused solely on males (Bossù et al., 2012). In addition, rats were tested during the period of adolescence instead of adulthood which is also different than several studies (Harré et al., 2008; Turrin et al., 2001; Walker et al., 2009). It was worth examining whether there were differences between SAL and LPS animals at P31 (21 days after exposure) and at P45 after behavioural testing to determine the effect of LPS alone and to be able to compare the effect when a mTBI is added later.
1.9.1 Differences in Cytokine mRNA levels in the HYPO at P31 after Neonatal LPS Exposure

Within the hypothalamus differences in cytokine mRNA levels were predominantly related to sex differences with females having higher levels of all cytokines examined compared to male rats (F=3.303, p<.01) (Figure 9). However, there was no difference between SAL and LPS animals for either sex.

Figure 9. Hypothalamus cytokine mRNA TNF-α, IL-1β, IL-6, and IL-10 at P31 differs as a function of sex but not neonatal exposure to LPS at P10.

Females had higher mRNA levels of all cytokines compared to males. n=4 for all groups. (###: p<.01).
1.9.2 Differences in Cytokine mRNA levels in the PFC at P31 after Neonatal LPS Exposure

There were no significant LPS exposure effects for either sex on TNF-α, IL-1β, IL-6, or IL-10 mRNA. Overall, there was a sex effect for the PFC, however unlike the hypothalamus males had higher expression of mRNA compared to females ($F=2.713, p<.05$) (Figure 10).

![Figure 10](image)

**Figure 10. Prefrontal cortex (PFC) mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 varies between male and female rats sacrificed on P31.**

Overall males had higher mRNA levels of all cytokines compared to females. n=4 for all groups. ($#: p<.05$).
1.9.3 Selection of Dorsal or Ventral Hippocampus

Both hippocampal regions were harvested separately at sacrifice, as they have been shown to have different anatomical connections and functional roles (Fanselow and Dong, 2010). The dorsal hippocampus is thought to be more responsible for memory and spatial processing with the ventral section primarily responsible for the emotional and fear responses associated with memories (Fanselow and Dong, 2010). To first determine if there were significant differences between the two regions in this model, a test plate examining brain derived neurotrophic factor (BDNF) mRNA expression was run. BDNF was selected as the marker is quite sensitive to neurodegeneration and has previously been examined in this model of mTBI (Mychasiuk et al., 2015a). There was no significant difference found between the two regions (F=0.519, p=0.478). In addition, since deficits (discussed below) seen in LPS + mTBI animals were primarily seen in anxiety measures such as the open field and forced swim, the VHPC was focused on because of its described connections and role in general emotive aspects and memory function.
1.9.4 Differences in Cytokine mRNA levels in the VHPC at P31 after Neonatal LPS Exposure

Unlike the HYPO or PFC, the VHPC demonstrated a difference between SAL and LPS Sham rats. Male LPS animals had higher expression of IL-6 mRNA compared to male SAL Shams (t=4.254, \( p<.01 \)) (Figure 11). A similar trend was observed in females but was not found to be significant. In addition, similar to the HYPO and PFC, there was a sex effect for IL-10 in the VHPC with males having higher levels of expression compared to female animals (F=5.472, \( p<.05 \)) (Figure 11).

![Bar graph showing cytokine expression in VHPC](image)

**Figure 11.** Neonatal LPS increased male IL-6 mRNA increase in ventral hippocampus (VHPC) on P31.

Male rats had higher levels of IL-10 in the VHPC compared to females. There is increased IL-6 expression for male LPS Shams compared to SAL Shams. n=4 for all groups. (#: \( p<.05 \), **: \( p<.01 \)).
1.10 Cytokine mRNA gene expression changes at P45 after LPS exposure

1.10.1 Differences in Cytokine mRNA levels in the HYPO at P45 after Neonatal LPS Exposure

After the conclusion of behavioural testing at P45, LPS and SAL Sham animals were sacrificed. This group allows for the determination of how the two groups responded to the stress of the behavioural testing between P31 and P45 as well as examining the impact of the neonatal exposure to LPS or SAL. Overall LPS animals had higher mRNA levels of the chosen cytokines compared to SAL animals \( F=6.795, p<.05 \). Individually, there is only a significant increase in IL-10 mRNA in female LPS compared to SAL controls \( t=3.298, p<.05 \). In addition, females also have a higher level of expression compared to males \( F=9.610, p<.01 \).

![Graph showing relative gene expression](image)

Figure 12. Increase in LPS female IL-10 mRNA in the hypothalamus (HYPO) for rats sacrificed on P45.

Females had higher levels of IL-10 mRNA compared to males. \( n=4 \) for all groups. (##: \( p<.01 \), **: \( p<.01 \), *: \( p<.05 \)).
1.10.2 Differences in Cytokine mRNA levels in the PFC at P45 after Neonatal LPS Exposure

Similar to the hypothalamus, LPS animals had overall higher mRNA expression compared to SAL animals (F=4.539, p<.05), however individually there was only a significant difference for female IL-1β mRNA levels (t=3.064, p<.05), with LPS animals having higher expression compared to SAL controls. There are no significant differences between males and females.

Figure 13. Increased mRNA expression of IL-1β in the prefrontal cortex (PFC) for female LPS rats sacrificed on P45.

There are no other significant differences due to LPS or sex. n=4 for all groups. (*) p<.05.
1.10.3 Differences in Cytokine mRNA levels in the VHPC at P45 after Neonatal LPS Exposure

Similar to results in the PFC, female LPS rats had higher levels of IL-1β mRNA at P45 compared to SAL controls ($t=3.400, p<.05$), with TNF-α following a similar trend but not significant. There are no difference between females and males for cytokine levels at P45 in the ventral hippocampus.

Figure 14. Increased mRNA expression of IL-1β in the ventral hippocampus (VHPC) for female LPS rats sacrificed on P45.

There is a significant increase in IL-1β mRNA for females that were exposed to LPS compared to SAL. n=4 for all groups. (*: $p<.05$).
1.11 Discussion of Cytokine Changes from LPS exposure

The predominant effects seen at 24 hours after mTBI (P31) between SAL and LPS rats are regionally dependent sex effects. This adds to the growing literature to suggest that males and females respond differently to immune responses and those changes may be regionally specific. There are two developmental windows where sex differences have been well characterized: prenatally and during the course of puberty (Juraska et al., 2013). The rats used in this study were between P31 – P45, which is still during an early phase of adolescence and at the beginning of sexual maturity (McCormick and Green, 2013). However even at this point there could be differences in basal cytokine mRNA levels between males and females. This work highlights the importance of how even in an acute-phase of injury males and females respond differently and sex-differences should be examined when possible.

The only significant change found at P31 from the LPS exposure was in the ventral hippocampus, an area that has previously been shown to be susceptible to modifications from an early life immune challenge (Harré et al., 2008). There are several explanations for this increase; there could be a regionally dependent persistent increase in IL-6 seen in male LPS Shams, however as LPS has previously been shown to cause only a short-term elevation of cytokine (Skelly et al., 2013; Turrin et al., 2001) this seems unlikely. An alternative explanation is that the LPS exposure sensitizes microglial cells (Williamson et al., 2011) and this is compounded briefly by the exposure to anesthetic. Isoflurane and other anesthetics have been shown to increase pro-inflammatory cytokines (Wu et al., 2012) and this could act as a mild ‘second hit’ and result in an increase in pro-inflammatory cytokines.
As the majority of these results show no changes between SAL and LPS exposed animals, it supports the idea that an early life exposure to LPS does not alter behavioural or molecular outcomes by itself. This is consistent with previous work in the lab showing that there are few overt behavioural changes from LPS alone to elevated plus maze or sucrose preference behaviour (Kentner et al., 2010; Spencer et al., 2006).

The exposure to LPS alone conferred little behavioural or mRNA expression changes at P31. However, when the cytokine results from P45 after behaviour testing are examined, several trends emerge. First, LPS animals have an overall higher expression of cytokines in the HYPO and PFC compared to SAL controls. However, when specific cytokines were analyzed only female LPS rats showed significant differences: IL-10 mRNA in the HYPO and IL-1β mRNA in both the PFC and VHPC. Since there is a rise in both pro and anti-inflammatory cytokine mRNA at P45 and they are within different brain regions it is unclear if there is any functional significance in the increases. It is interesting to note that females showed greater changes in gene expression than males at P45. This susceptibility could be because females are becoming sexually mature and it is possible that estrogen and progesterone could be altering their cytokine response (Johnson et al., 2006; Pyter et al., 2013). However, it could also be due to a sex-dependent response to the neonatal LPS and subsequent behavioural battery; this will be discussed as a limitation in Section 1.24.
1.12 *Aim Two: Examining the effects of mTBI alone*

1.12.1 *Rationale*

This experiment utilized the weight drop model characterized previously in this lab (Mychasiuk et al., 2015b, 2014a, 2014b). However, to ensure consistency between methodology and outcomes, the same behavioural test battery was performed. As these rats were exposed to an injection on P10 that could act as a form of early life stress, it was important to make sure that rats experienced behavioural changes that matched previous work. In addition, one group of animals was sacrificed at 24 hours after mTBI to examine the acute effects of the mTBI on peripheral and regional mRNA levels of TNF-α, IL-1β, IL-6, and IL-10.
1.12.2 *Time-to-right and Beam Walking (P31)*

There were significant increases in the time-to-right as a function of the mTBI animals compared to controls for both males and females (F=30.25, *p*<.001) (Figure 15A). Overall, mTBI animals also showed a greater number of foot slips across the beam (F=24.97, *p*<.001) and this was more predominant in males (t=3.800, *p*<.001) compared to females (t=3.274, *p*<.01) (Figure 15B). Lastly, there was an overall increased average time-to-cross the beam (F=5.266, *p*<.05) but this was significant in males alone (t=3.548, *p*<.01) (Figure 15C).

**Figure 15. Increased motor dysfunction following mTBI on P30.**

A) mTBI animals took longer to resume a supine position compared to Shams. B) mTBI animals had an increased number of foot slips across the beam compared to Sham animals. C) There was an increased average time-to-cross for males that received a mTBI compared to Shams. Females: Sham n=12, mTBI n=11 and for males: Sham n=16, mTBI n=12. (**: *p*<.01, ***: *p*<.001).
1.12.3 Open Field (P32)

There was no change in the distance rats moved in the open field after they experienced a mTBI. However, female rats tended to move a greater distance compared to males ($F=10.81$, $p<.01$). There was no change in the amount of time spent in the center of the open field between mTBI and Sham animals ($p>.05$). However, similar to the overall distance moved, females spent more time in the center of the field compared to males ($F=6.276$, $p<.05$).

![Graph A: Distance comparison between females and males, showing a significant difference for females.](image1)

![Graph B: Time spent in center comparison between females and males, showing a significant difference for females.](image2)

**Figure 16. No deficits in open field activity from mTBI alone.**

A) Females travelled a greater distance during the ten minute trial compared to males. B) Females spent more time in the center of the open field compared to males. Females: Sham n=8, mTBI n=7 and for males: Sham n=12, mTBI n=8. (#: $p<.05$, ##: $p<.01$).
1.12.4 *Elevated Plus Maze (P33)*

There was a significant decrease in the amount of time male mTBI rats spent in the closed arms of the maze compared to sex-matched controls ($t=2.278$, $p<.05$). Female mTBI and Sham animals did not differ significantly for the time spent in the closed arms. Females spent more time in the open arms compared to males but there was no effect of injury ($F=4.166$, $p<.05$).

![Figure 17](image.png)

**Figure 17.** Male mTBI animals spent less time in the closed arms of the elevated plus maze.

A) Male mTBI rats spent less time in the closed arms of the maze compared to Shams. B) Female rats spent more time in the open arms compared to males. Females: Sham n=8, mTBI n=7 and for males: Sham n=12, mTBI n=8.  (#: $p<.05$, *: $p<.05$).
1.12.5 Novel Context Mismatch (P35 -38)

Overall rats with a mTBI performed more poorly in the novel context mismatch, as characterized by a decreased preference for the novel object (% time with novel object) compared to Shams (F=15.09, p<.001). When post-hoc analysis was done, this effect was only significant for female mTBI animals, which showed a decrease (t=3.719, p<.01) compared to Shams. Males show a similar trend but it was not significant. There was no change in the amount of time spent exploring the environment.

![Figure 18](image)

**Figure 18. Female mTBI rats showed working memory deficits in the novel context mismatch paradigm.**

A) Females show a decrease in their preference for the novel object. Males follow a similar pattern but it was not significant. B) There are no changes in exploratory time during the trial between Sham and mTBI animals. Females: Sham n=8, mTBI n=7 and for males: Sham n=12, mTBI n=8. (**: p<.01).
This play comparison was done between a pair of SAL Sham + SAL Sham rats and a pair of SAL+ mTBI and LPS + mTBI rats. Due to the nature of the caging, there were no SAL + mTBI + SAL mTBI pairings to be examined. Overall the animals in the mTBI conditions demonstrated fewer rotations and a greater number of evasions or avoidance behaviour. However, only the decrease in rotations for male mTBI rats was significant ($t=3.531, p<.01$).

**Figure 19.** Male mTBI paired animals showed fewer rotations during play compared to Sham animals.

A) There is no significant difference in the number of attacks between Sham paired and mTBI paired animals. B) There was a decrease in the percent of rotations for male mTBI animals. C) There was no significant change between Sham and mTBI paired animals for avoidance behaviour. Four pairings of each group were examined. (**: $p<.01$).
1.12.7 Forced Swim (P44)

Overall, male rats spent more time immobile compared to females (F=23.02, \(p<.001\)). In terms of the effect of mTBI, male rats spent more time immobile compared to Shams (\(t=5.502, p<.001\)) while female mTBI animals actually spent less time immobile compared to Shams. There was a significant interaction suggesting that male and females are responding in an opposite manner to the injury (F=21.96, \(p<.001\)).

![Bar chart showing time immobile for males and females with mTBI and Sham groups.](image)

**Figure 20.** Male rats that underwent mTBI spent more time immobile compared to Sham rats.

There was a significant interaction in the response between male and female mTBI animals with females spending less time immobile and males spending more time immobile. Females: Sham n=8, mTBI n=7 and for males: Sham n=12, mTBI n=8. (###: \(p<.001\), ***: \(p<.001\)).
1.12.8 *Brain and body weight (P31 and P45)*

There were no changes in brain or body weight at P31 from either sex or injury. However, similar to results from Aim One at P45 males had heavier brains and body weights compared to females (F=4.687, p<.05, and F=5.887, p<.05 respectively).

![Graphs showing brain and body weight at P31 and P45 after mTBI.](image)

**Figure 21. Brain and body weight at P31 and P45 after mTBI.**

A) There was no difference in the brain weight between or within groups. B) No difference in body weight. N=4 for males and females. C) Males had heavier brains than females at P45. D) Males had heavier body weights at P45 compared to females. Females: Sham n=8, mTBI n=7 and for males: Sham n=12, mTBI n=8. (#: p<.05).
1.13 Discussion of Behavioural Changes after mTBI

Overall after mTBI there were changes in motor behaviour, anxiety, working memory, social behaviour, and immobile time that closely resemble previous results from our lab (Mychasiuk et al., 2014a). The behavioural tests demonstrate early effects on motor function, after the injury with an increased number of foot slips across the beam. In addition, there were memory deficits in the females (although males followed the same pattern) and depressive-like behaviour as measured by immobile time in the forced swim task for male rats. There were some alterations in the play behaviour seen such as a decreased number of attacks and rotations seen in mTBI paired animals compared to Shams.

However, there was no significant difference in the open field as described previously in our lab (Mychasiuk et al., 2014a). This could be due to the slight variation in this experiment, as all rats were exposed to an early life stressful injection of either LPS or SAL which may have affected how the animals performed (Deutsch-Feldman et al., 2015). In addition, some of the previous literature varies the length of time the animals spend in the open field. Lastly, mTBI is a heterogeneous injury and this particular group of animals might be more variable than previous groups examined.

In both elevated plus maze and the forced swim test, impairments were found in the performance for male rats only. However for the novel context mismatch, female mTBI showed greater impairments than males. The differences between males and females could indicate a slightly differential injury response. There is preliminary research that suggests that males and females might respond differently after mTBI (Broshek et al., 2009).
1.14 Molecular cytokine changes 24 hours after mTBI

The inflammatory markers that have previously been studied have primarily relied on using CCI or FPI models and even between those two models there are inconsistencies (Xiong et al., 2013). The predominant findings previously have shown a pro-inflammatory state as demonstrated by increased levels of TNF-α and IL-1β approximately 4 – 6 hours after the injury. The time point of 24 hours was taken for the reason that this was determined to be a peak time where there was entry of leukocytes through the BBB (Soares et al., 1995). In this study, cytokines and serum samples were measured to determine an acute response following the weight-drop injury. To test peripheral markers, protein levels measured by ELISA assays were tested on serum samples. In addition, mRNA levels of TNF-α, IL-1β, IL-6, and IL-10 in the hypothalamus, prefrontal cortex, and ventral hippocampus were evaluated to determine whether there were regional changes after mTBI.

1.14.1 Serum samples at 24 hours after mTBI

Serum samples that were collected at 24 hours after mTBI were tested using standard, commercial ELISA plates for cytokines TNF-α, IL-1β, IL-6 and IL-10. All samples regardless of sex, injury, or neonatal exposure were found to have concentrations below the lowest standard on the curve. To ensure the plate was properly coated and working, positive controls were included on each plate. Each positive control was read within the expected parameters included on the kit. The average absorbance (+/- SEM) of serum samples are represented in Tables 1 and 2. Concentrations were not calculated because the values would be off the standard curve and therefore there would be a high degree of error. Overall these results suggest that there was no
detectable increase of the peripheral cytokine levels 24 hours after injury in any of the groups (SAL + Sham, LPS + Sham, SAL + mTBI, and LPS + mTBI).

Table 1. Absorbance at 450nm for female serum samples tested for TNF-α, IL-1β, IL-6 and IL-10 24 hours after mTBI. All samples read at or below the lowest standard for the selected cytokine.

<table>
<thead>
<tr>
<th>Experimental Group - Females</th>
<th>ELISA Cytokine Absorbance P31</th>
<th>SEM</th>
<th>SEM</th>
<th>SEM</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL Sham</td>
<td>TNF-α</td>
<td>0.082</td>
<td>0.001</td>
<td>0.088</td>
<td>0.003</td>
</tr>
<tr>
<td>LPS Sham</td>
<td>IL-1β</td>
<td>0.093</td>
<td>0.003</td>
<td>0.113</td>
<td>0.007</td>
</tr>
<tr>
<td>SAL mTBI</td>
<td>IL-6</td>
<td>0.085</td>
<td>0.002</td>
<td>0.101</td>
<td>0.006</td>
</tr>
<tr>
<td>LPS mTBI</td>
<td>IL-10</td>
<td>0.084</td>
<td>0.001</td>
<td>0.087</td>
<td>0.002</td>
</tr>
<tr>
<td>Lowest Standard</td>
<td></td>
<td>0.143</td>
<td>0.000</td>
<td>0.149</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Table 2. Absorbance at 450nm for male serum samples tested for TNF-α, IL-1β, IL-6 and IL-10 24 hours after mTBI. All samples read at or below the lowest standard for the selected cytokine.

<table>
<thead>
<tr>
<th>Experimental Group - Males</th>
<th>ELISA Cytokine Absorbance P31</th>
<th>SEM</th>
<th>SEM</th>
<th>SEM</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL Sham</td>
<td>TNF-α</td>
<td>0.081</td>
<td>0.002</td>
<td>0.092</td>
<td>0.001</td>
</tr>
<tr>
<td>LPS Sham</td>
<td>IL-1β</td>
<td>0.088</td>
<td>0.003</td>
<td>0.094</td>
<td>0.002</td>
</tr>
<tr>
<td>SAL mTBI</td>
<td>IL-6</td>
<td>0.085</td>
<td>0.001</td>
<td>0.093</td>
<td>0.002</td>
</tr>
<tr>
<td>LPS mTBI</td>
<td>IL-10</td>
<td>0.090</td>
<td>0.003</td>
<td>0.103</td>
<td>0.001</td>
</tr>
<tr>
<td>Lowest Standard</td>
<td></td>
<td>0.143</td>
<td>0.000</td>
<td>0.149</td>
<td>0.008</td>
</tr>
</tbody>
</table>

65
1.14.2 Differences in Cytokine mRNA levels in the HYPO 24 hours after mTBI

Within the hypothalamus, there were no significant changes between Sham and mTBI animals for either males or females. Female rats had higher levels of IL-10 mRNA compared to male rats ($F=9.725, p<.01$). Female mTBI animals had a trend of lower levels of expression for IL-10, compared to Sham controls, but this was not significant ($p>.05$).

![Figure 22](image)

**Figure 22.** mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the hypothalamus (HYPO) at P31 after mTBI.

Females had higher expression of IL-10 mRNA compared to male rats; however there was no significant difference between Sham and mTBI animals. n=4 for all groups. (##: $p<.01$).
1.14.3 Differences in Cytokine mRNA levels in the PFC 24 hours after mTBI

There were no significant differences in cytokine mRNA levels between Sham and mTBI animals in the prefrontal cortex 24 hours after injury. In addition, there were no significant differences between males and females for these groups, although particularly in males there was a considerable amount of variation.

![Graph showing mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P31 after mTBI.](image)

**Figure 23.** mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P31 after mTBI.

There is no significant difference between Sham and mTBI animals 24 hours after mTBI. n=4 for all groups.
Differences in Cytokine mRNA levels in the VHPC 24 hours after mTBI

Within the ventral hippocampus, higher levels of TNF-α (t=2.510, p<.05) and IL-1β (t=3.149, p<.05) mRNA were found in female mTBI animals, as compared to controls, 24 hours after mTBI. There were no significant differences for male mTBI rats compared to Shams, nor were there any significant sex-dependent differences.

Figure 24. Increases in TNF-α and IL-1β for females rats in the ventral hippocampus (VHPC) at P31 after mTBI.

There were significant increases in TNF-α and IL-1β mRNA for female mTBI rats compared to Shams. There was no change in expression in male rats. n=4 for all groups. (*: p<.05).
1.15 *Molecular cytokine changes at P45 after P30 mTBI*

1.15.1 *Serum Cytokine Levels at P45*

Similar to results at P31, there were no detectable differences in peripheral cytokines (TNF-α, IL-1β, IL-6 and IL-10) levels at P45. All samples regardless of sex, injury, or neonatal exposure were found to be below the lowest standard on the curve. Again, to ensure the plate was properly coated and working, positive controls were included on each plate. Each positive control was read within the expected parameters included on the kit. The average value for the absorbance at 450 nm for the unknown serum samples is displayed in Table 3 and 4. Because the values read below the lowest standard, no conversion to concentration is made, normally a diluted standard curve would be done to get concentration levels; however, this was not necessary as the ELISA’s show that all cytokine levels are below normal detection range.

<table>
<thead>
<tr>
<th>Experimental Group - Females</th>
<th>ELISA Cytokine Absorbance P45</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>SAL Sham</td>
<td>0.082</td>
</tr>
<tr>
<td>LPS Sham</td>
<td>0.085</td>
</tr>
<tr>
<td>SAL mTBI</td>
<td>0.075</td>
</tr>
<tr>
<td>LPS mTBI</td>
<td>0.076</td>
</tr>
<tr>
<td>Lowest Standard</td>
<td>0.118</td>
</tr>
</tbody>
</table>

Table 3. Absorbance at 450nm for female serum samples tested for TNF-α, IL-1β, IL-6 and IL-10 taken at P45. All samples read at or below the lowest standard for the selected cytokine.
Table 4. Absorbance at 450nm for male serum samples tested for TNF-α, IL-1β, IL-6 and IL-10 taken at P45. All samples read at or below the lowest standard for the selected cytokine.

<table>
<thead>
<tr>
<th>Experimental Group - Males</th>
<th>ELISA Cytokine Absorbance P45</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>SAL Sham</td>
<td>0.075</td>
</tr>
<tr>
<td>LPS Sham</td>
<td>0.074</td>
</tr>
<tr>
<td>SAL mTBI</td>
<td>0.087</td>
</tr>
<tr>
<td>LPS mTBI</td>
<td>0.073</td>
</tr>
<tr>
<td>Lowest Standard</td>
<td>0.118</td>
</tr>
</tbody>
</table>
1.15.2 Differences in Cytokine mRNA levels in the HYPO 15 days after mTBI

At P45, after the behavioural testing, we found overall higher levels of mRNA in mTBI rats as compared to controls (F=16.50, p<.001). In terms of a more specific analysis, female mTBI rats had higher expression of TNF-α mRNA compared to both Sham (t=3.046, p<.05) controls and males (F=9.365, p<.01). Males had higher levels of IL-1β mRNA after mTBI and behaviour compared to Sham animals (t=3.565, p<.01).

![Sex-dependent increase in TNF-α and IL-1β mRNA in the hypothalamus (HYPO) at P45 after mTBI (P30).](figure)

Overall ANOVA results showed that mTBI animals had higher levels of cytokine expression compared to Shams. There were significant increases for TNF-α mRNA in female mTBI rats and IL-1β mRNA in males mTBI rats compared to Shams. n=4 for all groups. (*: p<.05, **: p<.01).
1.15.3 Differences in Cytokine mRNA levels in the PFC 15 days after mTBI

Similar to in the hypothalamus, mTBI animals had higher levels of cytokine mRNA expression at P45 compared to Sham controls (F=8.095, p<.01). There were however, no significant differences between males and females in the PFC (p>.05).

![Graph showing mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the PFC at P45 after mTBI.]

**Figure 26.** mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P45 after mTBI.

Overall ANOVA results showed that mTBI animals had higher levels of cytokine expression compared to Shams. n=4 for all groups.
1.15.4 Differences in Cytokine mRNA levels in the VHPC 15 days after mTBI

There were no significant changes between Sham and mTBI animals in the VHPC at P45. There was a similar trend as the HYPO and PFC with mTBI having higher levels of expression of cytokines, but this was not significant in this region (\(p=.144\)). There are no significant differences between males and females.

Figure 27. mRNA expression of TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-10 in the ventral hippocampus (VHPC) at P45 after mTBI.

There were no significant differences between Sham and mTBI animals. n=4 for all groups.
1.16 Discussion of Differences in Cytokine Profiles following mTBI

In regards to changes in peripheral protein markers, it appears that the selected cytokines are not useful biomarker measures in this particular model of mTBI. No significant difference between experimental groups was measured, and all groups had low peripheral levels that were either at or below the lowest measurable standard. Previous work has shown that there are only elevated peripheral cytokines after severe injury or within cerebral spinal fluid; in addition the timing is variable and ranges from increasing several hours after injury to days or weeks after the event (Zetterberg et al., 2013).

In this study we found relatively few changes in cytokines 24 hours after mTBI. These results are consistent with others that demonstrate short-lived cytokine levels in most TBI models, and thus 24 hours might be too late after injury to measure acute changes (Shohami et al., 1994; Xiong et al., 2013). In addition significant differences may be due to variability between mTBI models. The model used in this study relies on a light anesthetic, a freely moving head and mimics most forms of mTBI that would be received from a sports related concussion. This is different from the FPI model or CCI that result in more focal and severe injuries (Marciano et al., 2002). As previously discussed both the FPI and CCI rely on long-term anesthetic, fixed skull, often a craniotomy, and higher rate of mortality (Xiong et al., 2013).

Similar to results found at P45 with LPS and SAL exposed animals, the animals that were exposed to a stressor (in this case mTBI) had increased levels of select cytokines at P45. However, this was only significant within the hypothalamus for TNF-α for females and IL-1β for males. Although not universal, this could suggest that if an animal is subjected to mTBI or LPS and then behavioural tests, the pre-existing condition attributed to higher cumulative stress
compared to control animals. However, efforts were made to prevent behavioural testing from being stressful such as a maximum test of 10 minutes, organizing tests from least to most stressful, and recovery days (no testing), within the testing period. All the animals tested were handled from birth so that they would become familiar with the experimenter. However, tests such as play (isolating overnight) and forced swim are stressful tests and could be having an acute effect on cytokines (Connor et al., 1997; Detke et al., 1995; Vecchiarelli et al., 2016). However, even rats that are subjected to chronic stressors show full recovery at 24 hours (Girotti et al., 2011). As rats in this study were subjected to mild intermittent stressors and not chronic severe stress there may be an underlying switch in their stress response. Typically a beneficial stress response is short-lived, with animals responding to the change in homeostasis and then returning to baseline. Perhaps the underlying exposure to neonatal LPS or mTBI injury exacerbates the behavioural stress (Espinosa-Oliva et al., 2011) and contributed to cytokine differences between these animals and controls.

Although a typical inflammatory response is short-lived, a mild sterile brain injury might provoke an extended inflammatory response (Almeida-Suhett et al., 2013). Recent imaging studies have shown that there is altered glucose metabolism, with increased or decreased uptake in select regions for up to 3 months after mTBI (Vállez García et al., 2016). The immune system is energetically expensive, and the production of pro or anti-inflammatory cytokines relies on different microglia metabolic states (Hanisch, 2002; Orihuela et al., 2015). The increased demand in glucose metabolism could be due to low but chronic levels of activated microglia. Indeed they have found that microglia maintain a persistent state of activation for a month after injury (Fenn et al., 2014). The elevation of TNF-α mRNA for females and IL-1β mRNA for
males in the HYPO, as well as suggestive increases in the PFC and VHPC could indicate that although mild this model is still capable of producing a lasting increase in cytokine mRNA.
1.17 Aim Three: Changing system dynamics – LPS and mTBI in combination

1.17.1 Rationale

The last aim of this study was to determine if there was a significant difference between SAL and LPS exposed animals after mTBI, by comparing differences between rats in the SAL + mTBI group with those in the LPS + mTBI group. This work was the main goal of the study, and was designed to investigate why some people are at risk for developing PCS. Pre-existing factors that a person goes into the injury with could contribute to outcome and the literature examining these factors to outcomes in mTBI is incomplete. An early immune challenge has been shown to alter later response to stroke and susceptibility to seizure, in animal models, by making select regions in the brain vulnerable to a secondary hit from seizure or stroke (Galic et al., 2008; Spencer et al., 2007). This concept of immune programming could be a potential factor in the way people respond to mTBI.
1.17.2 *Time-to-right and Beam Walking (P31)*

There was no significant difference between SAL + mTBI and LPS + mTBI rats for any acute measures after mTBI such as time-to-right, the number of foot slips across the beam, or the average time to cross the beam.

![Graph showing time-to-right and number of foot slips](image)

**Figure 28.** No change in motor impairments or time-to-right following mTBI from LPS exposure for female or male rats.

A) No change in the time-to-right after mTBI for SAL or LPS rats. B) There was no change in motor deficits observed in mTBI rats from neonatal exposure to LPS. C) There was no change in the time-to-cross between SAL and LPS mTBI rats. Female rats n=11, and male rats n=12.
1.17.3 *Open Field (P32)*

There was no significant difference between rats that were exposed to neonatal SAL or LPS for the distance travelled during the open field trial. However, both female and male LPS + mTBI rats spent more time in the center of the open field compared to SAL controls (F=8.553, \(p<.01\)). Individually, the result was only significant for female rats (t=2.745, \(p<.05\)) which were spending approximately twice the time in the center of the field (Figure 29B).

**Figure 29.** Female LPS + mTBI rats spent a greater amount of time in the center of an open field.

A) There was no difference in the distance moved during the trial between SAL and LPS mTBI rats. B) Female LPS + mTBI rats spent more time in the center of an open field than SAL+ mTBI rats. (*) \(p<.05\). Female rats n=7, and male rats n=8.
1.17.4 Elevated Plus Maze (P33)

There was no significant difference in the amount of time the animals spent in the closed arm when sex and exposure to SAL or LPS were examined. Similarly, there was no significant difference in the amount of time spent in the open arms, although female LPS + mTBI rats tended to spend more time in the open arms compared to SAL + mTBI controls. In general, female rats also spent more time in the open arms compared to males (F=5.793, p<.05).

![Graph A](image)

**Figure 30.** No change in anxiety-like behaviour on the EPM from neonatal exposure to LPS and P30 mTBI.

A) There was no difference in the amount of time the animals spent in the closed arms. B) Females spent more time in the open arms compared to males. (#: p<.05). Female rats n=7, and male rats n=8.
1.17.5 Novel Context Mismatch (P35 – 38)

Both male and female LPS + mTBI rats spent less time with the novel object compared to SAL + mTBI rats (F=7.213, \( p<.05 \)). If analyzed separately, a significant difference (t=2.476, \( p<.05 \)) was only found in females suggesting a potential underlying sex effect. There was also a sex difference with males spending more time with the novel object compared to females (F=8.552, \( p<.01 \)). There was no difference in exploratory time between LPS and SAL mTBI rats.

Figure 31. Female LPS + mTBI rats showed greater deficits in working memory compared to SAL + mTBI rats.

A) The amount of time rats spent with a novel object was reduced significantly for female LPS + mTBI rats compared to SAL + mTBI. B) There was no significant difference in exploratory time between the groups. (##: \( p<.01 \), *: \( p<.05 \)). Female rats \( n=7 \), and male rats \( n=8 \).
1.17.6 Play (P41 – 42)

As the main question in this aim was to determine if there were differences between SAL + mTBI and LPS + mTBI animals, the play pairings were broken down by partner to determine if there was a difference in which animal was initiating play, rotations, evasions, etc. This approach allows the determination of which animal in an SAL + mTBI and LPS + mTBI pairing initiates play and the type of play that follows. Although not significant, female LPS + mTBI initiated play less, and had a greater number of rotations and evasions compared to their partner. Male groups were highly variable for the number of attacks and rotations. Overall there were no significant differences between SAL + mTBI and LPS + m TBI animals.

**Figure 32.** There was no difference in which partner of a SAL+ mTBI and LPS + mTBI initiates play or in the number of rotations or evasions that follow.

A) There were no differences in the number of times a partner initiates play. B) There were no significant differences in the number of rotations a partner performs. C) There was no difference in the amount of avoidance behaviour between SAL + mTBI and LPS + mTBI animals. Four play pairings were examined.
1.17.7 Forced Swim (P44)

In the forced swim test, female LPS + mTBI rats spent more time immobile compared to controls (t=3.633, p<.01). There was also a significant sex-effect with male rats spending more time immobile overall (F=8.052, p<.01). In addition, male and female rats responded differently to the combination of LPS + mTBI in that male LPS + mTBI rats spent less time immobile compared to their control groups, while female LPS + mTBI rats spent more time immobile compared to their controls (F=14.17, p<.001).

![Graph showing time immobile for females and males in response to LPS vs. SAL treatments.](image)

**Figure 33.** Female LPS + mTBI spent more time immobile in the forced swim task compared to SAL + mTBI controls.

Male rats spent more time immobile overall and the exposure to neonatal LPS had an interaction effect: females spent more time immobile and males spent less time immobile compared to SAL controls. (##: p<.01 (sex comparison), **: p<.01 (sex-matched control). Female rats n=7, and male rats n=8.)
1.17.8 Brain and Body Weight (P31 and P45)

There was no significant difference in brain or body weight at P431 or P45 between SAL or LPS mTBI rats. At P45, males had heavier brain (F=6.800, p<.05) and body weights (F=6.011, p<.05) compared to females.

![Figure 34. No difference in brain or body weight between SAL and LPS mTBI rats.](image)

A) No difference in brain weight at P31. B) No difference in body weight at P31. N=4 for females and males. C) Males had heavier brain at P45 compared to females. D) Males had higher bodyweight at P45 compared to females. (#: p<.05). Female rats n=7, and male rats n=8.
1.18 **Discussion of LPS + mTBI Behavioural Changes**

There were changes seen in measures of anxiety, memory, and depressive-like behaviours for LPS + mTBI rats compared to the SAL + mTBI controls. Spending more time in the center of the open field seems to indicate that female LPS + mTBI rats are less anxious compared to SAL + mTBI. Although the results that link neonatal exposure to later anxiety behaviour appears dependent on the timing of the exposure and the timing of testing for the older rat, this does match with results found previously by (Dinel et al., 2014). However, the observed reduction in measures of anxiety would seem unusual given that animals spent more time immobile in the forced swim task; a finding that is usually interpreted as greater depressive-like behaviour (Detke et al., 1995). Anxiety and depression have a high rate of co-morbidity in adolescence (Brady and Kendall, 1992) but the conditions can also occur simultaneously.

An alternative interpretation to the amount of time spent in the center of the open field is that perhaps these rats were more curious. There have been reports that neonatal exposure to LPS causes increased adolescent exploratory behaviour (Rico et al., 2010). Given the relatively long period of testing (10 minutes) it is possible that LPS + mTBI rats spent more time in the center of the open field because they explored slightly more. In the novel context mismatch paradigm, female LPS + mTBI rats explored both objects more than SAL + mTBI although this was not significant.

In addition to changes in emotional behaviour, female LPS + mTBI rats also spent less time with the novel object in the novel context mismatch paradigm. This finding is consistent with other studies that have indicated that an early age injection of LPS can alter memory performance after a second “hit” (Bilbo et al., 2008; Williamson et al., 2011). It is unclear why
these deficits are seen solely in females in our study, but there have been reports that females are more likely to develop PCS symptomology including memory impairment after mTBI (Ryan and Warden, 2003). Females might be more vulnerable to the effects of the combination of LPS and mTBI, while males in this study have shown to respond more strongly to mTBI alone.
1.19 Molecular changes at P31 after LPS + mTBI Rats

1.19.1 Differences in Cytokine mRNA levels in the HYPO at P31 after LPS (P10) and mTBI (P30)

There were few changes between SAL + mTBI and LPS + mTBI animals in the HYPO 24 hours after injury. However, there was a higher level of IL-6 mRNA expression in male LPS + mTBI rats (t=3.571, p<.05) compared to SAL+ mTBI. There were no sex differences between the groups (p>.05).

![Figure 35. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the hypothalamus (HYPO) at P31 for SAL + mTBI and LPS + mTBI rats.](image)

There was an increase in IL-6 mRNA for males LPS + mTBI rats compared to their control SAL + mTBI group. n=4 for all groups. (*: p<.05).
1.19.2 Differences in Cytokine mRNA levels in the PFC at P31 after LPS (P10) and mTBI (P30)

There were no significant changes between SAL+ mTBI and LPS + mTBI animals in the PFC at P31. However, it is worth mentioning that a trend was observed in male LPS + mTBI rats having lower levels of mRNA for the pro-inflammatory cytokines TNF-α and IL-6 compared to SAL + mTBI animals. Further, overall males had higher levels of IL-10 mRNA compared to females (F=5.860, p<.05).

Figure 36. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P31 for SAL + mTBI and LPS + mTBI rats.

There were no significant changes between SAL and LPS mTBI rats. Males had higher levels of IL-10 mRNA compared to females. n=4 for all groups. (#: p<.05).
1.19.3 Differences in Cytokine mRNA levels in the VHPC at P31 after LPS (P10) and mTBI (P30)

There was a significant decrease in TNF-α mRNA at P31 for female LPS + mTBI compared to SAL + mTBI ($t=2.561, p<.05$). There were no changes between the two groups for the rest of the cytokines examined at this time point, nor were there any differences in overall levels between the sexes.

Figure 37. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the ventral hippocampus (VHPC) at P31 for SAL + mTBI and LPS + mTBI rats.

A significant difference was found in TNF-α mRNA for female LPS + mTBI compared to SAL + mTBI rats. $n=4$ for all groups. (*: $p<.05$).
1.19.4 Differences in Cytokine mRNA levels in the HYPO at P45 after LPS (P10) and mTBI (P30)

Overall, in the hypothalamus LPS + mTBI rats had lower mRNA expression of the cytokine levels (TNF-α, IL-1β, IL-6, and IL-10) compared to SAL + mTBI animals (F=11.57, p<.01). Specifically, there were significant decreases in TNF-α mRNA in females (t=3.120, p<.05) and IL-1β mRNA in males (t=3.104, p<.05). There were no differences in expression between the sexes.

Figure 38. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the hypothalamus (HYPO) at P45 for SAL + mTBI and LPS + mTBI rats.

Overall lower expression for LPS + mTBI animals for the selected cytokines compared to SAL + mTBI animals. There were specific differences in TNF-α mRNA for females and IL-1β for males between LPS + mTBI animals as compared to their respective controls. n=4 for all groups. (*: p<.05).
Differences in Cytokine mRNA levels in the PFC at P45 after LPS (P10) and mTBI (P30)

A large amount of variation was found in the mRNA levels within each group in the PFC. There were no significant changes between female SAL + mTBI and LPS+ mTBI animals for any of the cytokine mRNA levels. However, in males TNF-α mRNA ($t=2.589, p<.05$) was lower for LPS + mTBI rats compared to controls. Further while IL-1β tended to be lower in the LPS + mTBI as well this was not significant.

![Graph showing mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P45 for SAL + mTBI and LPS + mTBI rats.](image)

There was a decrease in TNF-α mRNA for male mTBI rats if exposed to neonatal LPS compared to SAL controls. $n=4$ for all groups. ($*: p<.05$).

Figure 39. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the prefrontal cortex (PFC) at P45 for SAL + mTBI and LPS + mTBI rats.
1.19.6 Differences in Cytokine mRNA levels in the VHPC at P45 after LPS (P10) and mTBI (P30)

Within the VHPC levels IL-10 mRNA was lower for both female ($t=3.678, p<.01$) and male LPS + mTBI rats ($t=2.771, p<.05$) compared to controls. There was also a sex-difference in the expression of TNF-α mRNA within the VHPC with females having higher levels compared to males ($F=4.990, p<.05$).

![Figure 40](image)

Figure 40. mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 in the ventral hippocampus (VHPC) at P45 for SAL + mTBI and LPS + mTBI rats.

Significantly lower levels of IL-10 mRNA were found for both female and male LPS + mTBI animals compared to their controls. Females had higher levels of TNF-α mRNA compared to male rats. n=4 for all groups. (#: $p<.05$, **: $p<.01$, *: $p<.05$).
1.20 Discussion of Differences in Cytokine Profiles at P31 and P45 after LPS and mTBI

Similar to the previous results showing changes between SAL and LPS Shams, and Sham vs mTBI animals, there were relatively few changes at P31. There was a significant increase in IL-6 in male LPS + mTBI animals compared to SAL + mTBI in the hypothalamus. However, as there was an increase in IL-6 for male LPS + Shams at this time point, it is likely due to residual effects from the exposure to LPS and not due to mTBI. As mentioned previously, the lack of changes after mTBI could be due to the temporal timing of cytokine release. It is likely that 24 hours is either too far or close after the mTBI to detect any significant rise in cytokines (Marciano et al., 2002).

The results from P45 demonstrate that the LPS + mTBI animals demonstrate a unique attenuated response compared to either LPS or mTBI alone. When SAL animals are compared to LPS animals after behavioural testing at P45 there are relatively few changes, but if there are significant differences LPS animals have higher levels of expression. Similarly, if Sham and mTBI animals are compared at P45, mTBI animals had higher cytokine mRNA compared to Shams. When LPS and mTBI are combined these animals respond in the opposite manner at P45 with an attenuated cytokine response. The ‘double hit’ could be contributing to a dampened response; this mechanism could be due to alterations in selective channel excitability such as NMDA (Harré et al., 2008). This change could also be because the resident microglia/macrophage cells in the brain are not producing the same level of cytokine activity in comparison to animals that only received LPS or mTBI. LPS is capable of activating microglia within the brain and is also known to cause long-term changes that are only apparent after further
injury or stress (Espinosa-Oliva et al., 2011). Therefore, to test whether this was a contributing factor, several markers of microglia were examined further.

1.21 Examination of Microglia Markers

1.21.1 Rationale

From the nature of the cytokine changes it appears that while rats exposed to injury or LPS alone tended to exhibit a higher regional cytokine mRNA expression, the LPS + mTBI animals had lower responses compared to controls. It is unclear why there would be a blunted or inhibited response in these animals. However, a possible explanation could be that the LPS modifies the response characteristics of brain microglia which becomes evident after the second hit (mTBI) (Bilbo and Schwarz, 2009; Espinosa-Oliva et al., 2011; Karve et al., 2016). Therefore, two additional genes which are representative of different microglia activation state were examined after the cytokines. These markers are not exclusive to microglia and have also been characterized on macrophage cells (Lawrence and Natoli, 2011).

1.21.2 Arginase 1 (Arg1)

Arginase 1 (Arg1) is an enzyme that converts L-arginine into prolines and polyamines that play roles in tissue repair and matrix deposition (Cherry et al., 2014). A high expression level of Arg1 is characteristic of an anti-inflammatory state of microglia/macrophage activation, which coincides with increased production of anti-inflammatory cytokines such as IL-10. As mTBI has been associated with changes in metabolism, it is also important to mention that
microglia/macrophage cells producing anti-inflammatory cytokines are predominantly relying on an oxidative state of energy production (Orihuela et al., 2015).

1.21.3 Inducible nitric oxide synthase (iNOS)

Inducible nitric oxide synthase (iNOS) is an enzyme that catalyzes the production of nitric oxide from L-arginine. In comparison to endothelial (eNOS) or neuronal (nNOS), iNOS is primarily activated by cytokines and immune activation. iNOS is highly expressed in microglia/macrophage cells that have a designated pro-inflammatory state, coinciding with secretion of cytokines such as TNF-α, after an injury to the CNS (Cherry et al., 2014). In addition to cytokine production, high concentrations of nitric oxide (NO), the enzyme can inhibit mitochondria respiration by interfering with Cytochrome C (Orihuela et al., 2015). In comparison to Arg1, iNOS and the release of pro-inflammatory cytokines by microglia/macrophage cells is primarily characterized by a glycolytic state of metabolism. Since both Arg1 and iNOS convert L-arginine they can effectively compete against each other (Orihuela et al., 2015). By examining both markers, a preliminary understanding of the phenotypic state of microglia activation and therefore a difference in response profile between LPS + mTBI animals and SAL + mTBI animals could be examined.
1.21.4 Differences in Arg1 and iNOS mRNA levels at P31 after LPS (P10) and mTBI (P30)

To best determine if the microglia activation system had changed from the combination of neonatal LPS and mTBI, markers were normalized to Sham conditions. Therefore, for the purpose of the analysis and depicted below, if an mTBI animal had an expression level over 1.0 this coincided with higher levels of gene expression compared to Shams, while a value below 1.0 suggested lower expression levels compared to Shams. At P31 there was a trend for female LPS + mTBI animals to have lower expression compared to SAL + mTBI controls ($p=.12$) but there was a large amount of variability. Males had significantly higher levels of Arg1 ($F=11.703$, $p<.01$) and iNOS mRNA ($F=8.373$, $p<.05$) expression in the hypothalamus compared to females.

![Figure 41](image-url)

**Figure 41.** Figure depicts data at P31 presented as the relative change in mRNA levels (ratio of expression) of Arginine 1 (Arg1) and inducible nitric oxide synthase (iNOS), in animals receiving LPS or SAL at P10 and a mTBI at P30.

A ratio value of 1.0 would indicate no difference between controls or mTBI groups. Males had significantly higher levels of Arg1 and iNOS mRNA in the hypothalamus ($##: p<.01$, #: $p<.05$).
1.21.5 Differences in Arg1 and iNOS mRNA levels at P45 after LPS (P10) and mTBI (P30)

For female LPS exposed animals there were significant differences between groups for Arg1 ($t=3.385$, $p<.05$) and iNOS mRNA in the HYPO ($t=4.174$, $p<.01$) and in the VHPC ($t=3.650$, $p<.05$ and $t=7.768$, $p<.01$ respectively) compared to SAL controls. For males there were significant differences for Arg1 mRNA for the HYPO ($t=4.081$, $p<.01$) and PFC ($t=3.061$, $p<.05$), as well as for iNOS mRNA in the PFC ($t=3.471$, $p<.05$) and VHPC ($t=3.600$, $p<.01$).

![Figure 42. Figure depicts data at P45 presented as the relative change in mRNA levels (ratio of expression) of Arginine 1 (Arg1) and inducible nitric oxide synthase (iNOS), in animals receiving LPS or SAL at P10 and a mTBI at P30.](image)

A) Females LPS + mTBI rats were significantly different for Arg1 and iNOS mRNA in the HYPO and VHPC compared to SAL + mTBI females. B) Male LPS + mTBI rats showed decreased expression for Arg1 mRNA in the HYPO and PFC compared to SAL + mTBI controls. There was decreased expression for iNOS mRNA between LPS + mTBI and SAL + mTBI rats in the PFC and VHPC. (**: $p<.01$, *: $p<.05$).
1.22 Discussion of Arg1 and iNOS mRNA Changes

Arg1 and iNOS, similar to cytokines examined, showed high amounts of variability and little changes when examined at P31. In terms of sex differences, males had higher levels of Arg1 and iNOS in the hypothalamus whereas for most cytokines examined, females had higher levels of expression compared to males. This could indicate that acutely, males and females have slightly different responses for both cytokines and microglia/macrophage cells (Schwarz et al., 2012). When P45 changes are examined, the microglia/macrophage markers were in line with the pattern of changes seen with the cytokine analysis. Both showed a blunted response in LPS + mTBI animals compared to that of the SAL + mTBI. This is most clearly represented in Figure 42, as the relative increase in microglia/macrophage expression for SAL + mTBI animals, coincides with some increase in cytokine expression (TNF-α and IL-1β) at P45. Similarly, LPS + mTBI animals’ show decreased expression of Arg1 and iNOS compared to LPS + Shams, which also coincides with decreased cytokine expression. Interestingly, this difference was not present at P31, suggesting the effect could be delayed or exacerbated from stress. Therefore, it seems likely that when animals experience modifying effects from both LPS and mTBI combined with mild stress they have decreased levels of microglia activity and also cytokine production. Some studies have suggested that stress is a necessary factor for LPS induced alteration of microglia to occur and the combination of mTBI and behavioural testing could illicit this response (Espinosa-Oliva et al., 2011). However, this thesis work is not conclusive as microglia cells were not isolated and although they have been used as markers, Arg1/iNOS are not definitive proof of activation.
1.23 Thesis Discussion Summary

The first goal in this study was to determine if there were long-term changes in behaviour and cytokine markers after exposure to neonatal LPS alone. Overall, in previous studies LPS given during the critical window of development resulted in little overt changes in adult behaviour (Kentner et al., 2010; Spencer et al., 2006). However, if rats are tested in adolescence, similar to this study design results suggest decreased anxiety-like behavior changes in adolescence (Dinel et al., 2014). Thus it appears that the timing of exposure and testing is critical, as LPS given early (P3-5) produces more robust increases of anxiety-like behaviour (Walker et al., 2009). The choice to administer LPS at P10 is because this time point correlates to the end of the third trimester and birth in humans which is when the immune system is not fully developed but an infant is able to experience infections outside of the prenatal environment. This period is a time when the physiology of the GABAergic system is undergoing significant changes from net excitation to inhibition (Swann et al., 1989). Interrupting the development of this system could change the molecular response to later mTBI. Results in our study demonstrate that the majority of tests revealed no alteration in either anxiety or depressive-like behaviour between SAL and LPS Shams. It is unclear whether the impaired time to cross that we observed was due to increased anxiety in crossing the beam or due to enhanced exploration as has been shown by (Rico et al., 2010).

Although memory deficits were seen in female mTBI animals and became worse when LPS and mTBI were combined, there were no changes from LPS alone. This is unexpected as studies have shown that peripheral immune challenges in adults can alter memory performance (Barrientos et al., 2009). However, our findings are accord with reports that most long lasting
neonatal LPS effects are not apparent until triggered by a ‘second hit’ such as injury or stress (Bilbo and Schwarz, 2009; Espinosa-Oliva et al., 2011).

Similar to what was seen in the behavioural studies, data from the cytokine mRNA showed very little changes from the exposure to LPS alone. At P30 there was an increase in IL-6 mRNA for male rats while at P45, higher levels of IL-10 and IL-1β mRNA were found in females. Overall, the relative lack of behavioural and cytokine mRNA changes between the LPS and SAL animals at P31 suggest that exposure to neonatal LPS alone does not chronically alter cytokine mRNA expression. However, the interaction of LPS and behavioural stress cause a slight elevation of cytokine expression in female rats. As there are both pro and anti-inflammatory mRNA increases and few behavioural changes, the significance of these changes will need to be determined in additional studies.

Although there were few overt behavioural changes from LPS alone, there are multiple persistent changes following mTBI. The behavioural results in this study are consistent with previous results in our lab (Mychasiuk et al., 2014a, 2015b, 2014b) and suggest a persistent impairment in cognitive function and affect that is sex-dependent. Although the behaviour has been well characterized, analysis of inflammatory molecular markers had not been examined and therefore both serum and brain tissue were analyzed at P31 (24 hours after mTBI) and P45. In general, there was no consistent increase in either cytokine mRNA or peripheral protein levels, this may be due to the mild nature of the injury, as it causes no macroscopic damage or fatality in comparison to the FPI or CCI models (Mychasiuk et al., 2014a; Xiong et al., 2013). Although several models have examined brain tissue and peripheral biomarkers, they yield variable levels of each marker and different temporal profiles (Marciano et al., 2002; Zetterberg et al., 2013).
Although there are a wide range of behavioural deficits, the cytokine data are less clear at both 24 hours (P31) and after behavioural testing (P45). For example, there are regionally dependent increases in cytokine mRNA at P45 in mTBI animals, that were not found at P31. Previous work has found a persistent increase in inflammatory markers or alterations in metabolism resulting from mTBI (Fenn et al., 2014; Vállez García et al., 2016). This could be further exacerbated by the mild stressors present in the behavioural battery, as tests such as forced swim and play could increase brain and circulating cytokines (Connor et al., 1997; Detke et al., 1995; Vecchiarelli et al., 2016). One caveat with respect to the cytokine mRNA data is that evidence of mRNA increases does not necessarily indicate that they are accompanying alterations in protein.

The third and main goal of the study was to determine if there were significant differences between the outcomes of SAL + mTBI animals compared to LPS + mTBI rats. Although not universal, changes were found in both behaviour and mRNA levels for cytokine and microglia/macrophage marker expression. In particular, female LPS + mTBI rats spent more time in the exposed area (center of the arena), more time immobile during the forced swim task, and less time with a novel object compared to SAL + mTBI animals. This finding is consistent with other reports suggesting that females were more likely to develop PCS following mTBI (Ryan and Warden, 2003). Specifically these findings suggest that females might be more susceptible to changes in anxiety, working memory and depressive-like symptomology when exposed to an early injection of LPS + mTBI. This vulnerability could be due to differences in female hormones such as estrogen and progesterone (Sarkaki et al., 2013), but there is growing evidence to suggest that sex hormones are neuroprotective (Stein, 2001). Another possible explanation is that, between P30 – P45 females have more activated microglia compared to
males as demonstrated by (Schwarz et al., 2012) which could make them more susceptible to the mTBI, behavioural stress or a combination of the variables. In addition to some behavioural changes, there was a significant difference in the cytokine and microglia/macrophage response between SAL + mTBI and LPS + mTBI animals. In both LPS and mTBI alone groups, there was a rise in a pro-inflammatory cytokine at P45, however in LPS + mTBI animals there was a significant decrease in all cytokine mRNA measured within the hypothalamus. This could indicate a switch in system dynamics when the two events are combined, in opposition to when either is presented independently. This possibility is supported by the lower levels of both Arg1 and iNOS in LPS + mTBI animals compared to SAL + mTBI animals at P45. Since both Arg1 and iNOS are lowered in the LPS + mTBI group and not polarized to either an M1 or M2 phenotype, this could provide additional support that microglia respond simultaneously (Morganti et al., 2016). The long-term effects are surprising but are similar to a study that showed that microglia remain sensitized after TBI for a month following injury (Muccigrosso et al., 2016).

1.24 Future Directions and Limitations

There are several future directions which should be pursued after this work. The behavioural testing completed was thorough in testing the majority of symptoms that present after mTBI. However, some behavioural test results were inconsistent between sexes, such as memory deficits found for females and not males. To follow up these results, tests could be done to examine different aspects of memory such as the Morris Water Maze to test spatial orientation and memory. In addition, LPS may confer an underlying susceptibility in both the amygdala and
hippocampus (Galic et al., 2008; Spencer et al., 2007). Although the hippocampus was analyzed for mRNA levels of select cytokines and functional performance was tested with memory tasks, there was no analysis of the amygdala. The harvesting of the amygdala and addition of adding fear-conditioning to the test battery could provide further information on regional susceptibility.

In addition to expanding the behavioural analysis, the majority of molecular testing showed alterations at P45 after mild stressors (behavioural testing). As rats were put through a battery of tests, this has to be considered as a contributing factor. As some of these tests provoke a stress response, which could impact the immune response as well. For example forced swim in particular has been associated with changes in circulating and brain cytokines although this is regionally dependent (Connor et al., 1997; Detke et al., 1995; Vecchiarelli et al., 2016). In healthy animals the stress response is short-lived and doesn’t chronically affect animals. Even animals that have underwent chronically intermittently stress with cold temperatures still return to baseline pro-inflammatory levels within 24 hours (Girotti et al., 2011). As mentioned previously, rats in this study were tested for a maximum of ten minutes per day, and the stress of the tasks varied. To gauge if there were differences in the stress response of these animals, more direct evaluation of the stress axis could be done by examining cortisol levels as well as for regional changes in expression of glucocorticoid and mineralocorticoid receptors. This would allow the experimenter some insight into the stress axis and response in these animals. In addition, to ensure that the test battery was not a confounding variable, a future cohort could be examined that would receive LPS at P10, mTBI on P30 and no behavioural testing until sacrifice on P45 to provide controls for the sole effect of LPS or mTBI
The majority of molecular work was conducted on mRNA levels, which does not automatically equate to protein levels, and may therefore be limited in terms of functional implications. For additional support of cytokine changes, Western Blots or ELISAs with the appropriate antibodies could be done to measure protein levels in the brain regions examined. In addition, there are multiple other cytokines or chemokines that could contribute to injury pathophysiology such as anti-inflammatory cytokine IL-4 or pro-inflammatory INF-γ. Therefore, a microarray of cytokines and chemokines could be examined to determine if there are observable patterns of expression. Finally, the markers Arg1 and iNOS are not specific to microglia and could indicate global changes. To better determine the role of microglia to mTBI, cell cultures of isolated microglia could be harvested and tested for levels of cytokines and neurotrophic factors. This would allow for the specific role of microglia to be examined.

In addition, to expanding the molecular data examined the timing of these markers is important. One of the most surprising findings of this study was that there was no consistent change in cytokines 24 hours after mTBI. Although this is probably due to the mild hit produced in this model, a hit titration curve could be done. To appropriately test this idea, a small group of animals would be sacrificed at 1, 3, 6, and 12 hours after mTBI to determine if there are more acute changes after the hit. This could provide greater insight into the temporal profile of cytokine production in this particular weight-drop model of mTBI.

1.25 Conclusions

Overall the aim of this thesis was to determine if an early life immune challenge could contribute to risk or resilience towards developing PCS after mTBI. Results from the first two aims show that while LPS is relatively benign, mTBI is capable of producing significant
behavioural deficits. However, both LPS and mTBI show relatively few changes in cytokine mRNA as either P31 or P45. This could be due to the timing of the sacrifice and relatively mild injury and response to LPS. The third section of this experiment, demonstrated that there is a difference in LPS + mTBI rats both behaviourally and on a molecular level. Female LPS + mTBI rats show alterations in measures of anxiety, affect, and working memory compared to SAL + mTBI rats. In addition both males and females show a differential cytokine response after the combination of LPS + mTBI compared to either alone.

If these results were to translate accurately to a human population, it would mean that infants who suffered a bacterial immune challenge around birth may have a modified susceptibility/resilience risk to mTBI as children. In addition, females could be at greater risk for developing emotional alterations and memory problems after mTBI. The cytokine results seen were not consistent in all brain regions, and there are significant differences between males and females. In addition, a future experiment with animals that are subjected to both LPS and mTBI without behavioural testing would be beneficial in determining how much of this response is dependent on additional “life” stressors. Overall the combination of LPS + mTBI impacts outcomes as demonstrated by the behavioural deficits in females, and an altered cytokine expression which could be due to changes in microglia/macrophage cells. Although not conclusive, this work allows for some further insight as to why some individuals are at risk for developing and detrimental symptoms after mTBI.
REFERENCES


Probert, L., 2015. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience, Inflammation in Nervous System Disorders 302, 2–22. doi:10.1016/j.neuroscience.2015.06.038


Ramlackhansingh, A.F., Brooks, D.J., Greenwood, R.J., Bose, S.K., Turkheimer, F.E.,
   Kinnunen, K.M., Gentleman, S., Heckemann, R.A., Gunanayagam, K., Gelosa, G., Sharp,
Readnower, R.D., Chavko, M., Adeeb, S., Conroy, M.D., Pauly, J.R., McCarron, R.M., Sullivan,
   P.G., 2010. Increase in blood-brain barrier permeability, oxidative stress, and activated
   3530–3539. doi:10.1002/jnr.22510
Reid, A.Y., Riazi, K., Campbell Teskey, G., Pittman, Q.J., 2013. Increased excitability and
   molecular changes in adult rats after a febrile seizure. Epilepsia 54, e45–e48.
   doi:10.1111/epi.12061
Relton, J.K., Rothwell, N.J., 1992. Interleukin-1 receptor antagonist inhibits ischaemic and
   leads to heightened exploratory activity in adolescent rats. Behav. Brain Res. 215, 102–
   109. doi:10.1016/j.bbr.2010.07.001
Robbins, C., Daneshvar, D., Picano, J., Gavett, B., Baugh, C., Riley, D., Nowinski, C., Cantu, R.,
   Stern, R., McKee, A., 2014. Self-reported concussion history: impact of providing a
   doi:10.1080/09540260310001606692


doi:10.1016/j.jss.2013.03.075


doi:10.1038/nrneurol.2013.9


doi:10.1002/ana.23536
