Increased GFAP in Motor Cortex and Bimanual Coordination Deficits in a Rat (*Rattus Norvegicus*) Model of Repeated Pediatric Mild Traumatic Brain Injury

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Abstract

Brain injury induces brain change, which is modulated by sex and developmental age. We examined brain change in juvenile rats after repeated mild brain traumatic injuries. We hypothesized the rat pups would show graded deficits in behavioural performance and a graded increase in protein expression, both increasing with the number of impacts. Three-week-old rats received 1-3 concussions over three days, compared to sham controls without concussive impact. The wire-hanging task revealed a dose-like effect of injury, with performance worsening with the addition of each injury. Glial acidic protein level was highest in the two-injury group. Corticosterone was significantly different for sex and injury status, without an interaction. Our findings suggest repetitive concussion causes rapid changes in motor behaviour and the brain in juvenile rats. Even mild brain injuries require care and attention to avoid repeated injuries and an increase to long-lasting brain disruption.

Keywords: Plasticity, rat, juvenile, brain injury, concussion, inflammation, GFAP, stress, CORT

Chapter I. History of Brain Plasticity

The term *brain plasticity* has moved into mainstream language as lay people became fascinated with the flexibility of the brain. Brain plasticity is the brain's ability to respond to the environment and adapting for effective operations. The scientific definition of brain plasticity is the morphological and functional change within the brain, driven by internal and external experience. Internal and external experience includes everything happening within the body, and everything an individual experiences within their lifetime. The plastic response to experience may be temporary or long-lasting, adaptive or maladaptive, and vary based on brain area and an individual's sex and age. Plastic change can be measured through micro changes in protein expression, changes in neuronal structure, or alterations in behaviour. As we gain a deeper understanding of brain plasticity's flexible nature, it becomes clear there are many factors we do not yet understand. Building onto the foundation of our knowledge of how the brain changes in response to experience will aid in the understanding of the process of learning and memory, as well as the treatment of stroke, injury, and mental health issues.

The Early Years

Initial research attempting to understand brain function and mechanisms progressed slowly. Case studies of humans with brain lesions were used to understand the way behaviour changed after a specific brain area was damaged. Crichton-Browne (1879) did a post-mortem analysis with body and brain weights of men and women collected from asylums and infirmaries to find correlations with intelligence and mental illness and found lower weights in patients with amentia (severe congenital mental handicap) and dementia compared to healthy controls and patients with depression. Brain weights were later found to be an unreliable measure of intelligence, as many factors influenced variation in brain weights. Another method used to

better understand and treat mental illness and personality issues was lobotomy. Doctors and researchers graded behaviour and emotional functionality of institutionalized patients with schizophrenia before and after prefrontal lobotomy and found all twenty patients improved after surgery (Schrader & Robinson, 1945). Improvement included increased cheerfulness, compliance, and care for appearance, but often alongside mental deficits and loss of bladder control. Although this approach would now be considered recklessly misguided, it was a serious approach to understand the involvement of frontal lobes in complex thought and emotion at the time. Kolb (1949) advocated for researchers from many institutes to create a large dataset of lobotomies of varied size and area. In asylums, patients have various life experience, age, and aliments that could confound the results of lobotomy, but also provide an extensive and broad data set. Kolb's own work employed lobotomy to relieve psychosis, disturbing behaviour, and minor personality disorders, but his goal of collaboration was that each individual would contribute new information to complete the picture of mental illness and brain function by area.

Classifying how personality and behaviour changed after damage including lesions to specific areas of the brain would, lead researchers to conclusions about each brain area. In order to understand which brain areas were responsible for different aspects of behaviour and personality, research needed to be simple with experimental control. Basic research focuses on manipulating one variable at a time, allowing for direct comparisons of behaviour, blood, brain tissue leading to more confidence in discoveries.

Rodent Models

The use of animals in research empowered researchers to ask direct questions without the confounds involved when using human participants. Laboratory animals often provide for precise control of the environment and development, allowing for manipulation of individual

variables. Amphibians, rodents, and monkeys have been essential for developing an understanding of brain processes. Analyzing structure and function of neuroanatomy across species is an important aspect in translating research based on other animals for human comparison. Non-mammal vertebrates often have brain structures analogous to structures in mammals (Sayegh & Lajtha, 1989), as such these animals may be used to investigate basic function and dysfunction. Analogous structures among mammals allow for animal models of human disease and disorders. A substantial proportion of the literature on brain plasticity has resulted from research with rodents. Rodents are cost-effective, easy to house, breed, and handle, making them an ideal choice for many types of research. Despite this fact, there is still disagreement on analogous developmental time points leading to continued investigation. Rodent neurodevelopment involves similar processes, but the stages of neurogenesis, synaptogenesis, gliogenesis, and oligodendrocyte maturation happen much faster in rodents compared to humans (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). Development influences behaviour, brain structure and biochemistry. Researchers must be aware of the specific maturation time points, and give consideration to developmental stage in research designs.

To extend our understanding, neurodevelopmental researchers compare animals raised in different environments. The life and environment of wild animals is more stimulating than that of domestic animals. A comparison of wild animal brains to their domestic forms revealed increased brain weight and more prominent fissures in the wild type (Herre, 1958). This brain change could have been driven by environment, but a more precise comparison was needed. Another group of researchers raised animals with the opportunity for more stimulation or enriched environments compared to animals housed in standard laboratory cages had an increase in cholinesterase, indicating change in brain chemistry (Krech, Rosenzweig, & Bennett, 1960).

Cholinesterase (ChE) is an enzyme that was used in this early research to determine cognitive activity based on increases and decreases within specific brain areas. The compilation of neurobiological and behavioural research by Bennett et al. (1964) on brain change after exposure to enriched environments is the first example in the literature using the term *brain plasticity*. Bennett, Diamond, Krech, and Rosenzweig (1964) gathered conclusive evidence that the brain responds to the environment, then examined whether memory and learning occurs through chemical and anatomical/structural change. In search of physical memory, mice were injected with a disruptive antibiotic, puromycin, during multiple stages of memory consolidation, which revealed a relationship between memory and synaptic change (Flexner, Flexner, & Roberts, 1967). This finding provided proof that learning occurred through both physical and chemical brain change. How or why this synaptic change occurred in relation to novel memories was still unclear.

The visual system was an early target area used to investigate the flexible nature of the brain because manipulation is often not difficult. Visual deprivation for example, can be used with young animals and, after this deprivation, animals can be compared to those with typical stimulation to find differences in brain areas. Visually depriving newborn animals and comparing developmental outcomes with those developing with typical visual input is one way to observe experience-expectant brain change (Riesen & Aarons, 1959). The visual system will not properly develop without visual stimulation. This type of research has pointed to optic nerve fibers having the capacity to change and the capacity to change is restricted by the timing of developmental experience (Keating & Gaze, 1970). Cynader, Berman, and Hein, (1976) looked further into restrictions on plastic recovery after early life visual deprivation and found, after longer periods, less of the visual system could be recovered, but even after fifteen months of

deprivation the feline brain was still capable of adapting. After one year of complete visual deprivation, then six months of normally lit environment the feline brain can recover several orientation responses. This neural recovery is evidence for flexibility and limited brain plasticity beyond the earlier critical period. Discovering sensory adaptation in older visually deprived cats demonstrates the capacity for adaptation within the visual system outside of early development.

Adaptive change after neuronal loss or damage is a form of brain plasticity originally thought to only be possible in young animals, but not in the adult mammalian brain. After experimentally induced denervation of synapses, adjacent neurons are capable of forming new connections to neurons that would not typically be connected, in order to compensate for missing connections after neuronal loss (Raisman & Field, 1973). Cells forming novel connections demonstrate the brain's ability to adapt after tissue has been lost in an attempt to regain function. Miles and Fuller (1974) found rhesus monkeys could make adaptive adjustments for optimum functioning in the vestibulo-ocular system to compensate for experimental spectacles changing their visual field. The brain is flexible enough to adjust specific mechanisms to new sensory input in order to restore typical function.

Maturation

Although the brain is changing at a considerable rate during development, there are different time points in maturation that can be targeted to boost or hinder recovery. The Kennard Principle suggests there is a negative relationship with brain injury outcomes and age. This principle has caveats, but we have relied on it for a long time. Young mammals generally recover from brain injuries more readily than adults, but if the injury is in early infancy there are other factors at play. Rats with prefrontal cortex lesions recover better if the lesion occurred on postnatal day ten than postnatal day one or five (Kolb, 1987). Without a sufficient amount of

brain organization and structure formation, the brain cannot compensate effectively after damage. After removing the medial prefrontal cortex in rats at several ages (postnatal day 3, 6, 9, 15, and 30) younger animals had better behavioural outcomes than the older groups, but animals lesioned at postnatal day nine had the best overall recovery (Kolb, Petrie, & Cioe, 1995). The rat brain will more readily recovery from trauma at key developmental timepoints.

The adult brain is capable of physically changing to promote recovery after disruptive experience. Animal research allows for direct observation into recovery related brain change. Research using rats to model human neurological and behavioural processes are easily translatable. A crucial difference in rat and human development is the level of maturation at birth. Rats are born at an earlier stage in development than humans for example, rat eyes are closed at birth which is a time point consistent with human premature birth. Based on the developing immune systems rodents aged 1-3 days are comparable with pre-term humans from 23-32 weeks gestation (Holsapple, West, & Landreth, 2003). Access to this early stage in development allows researchers to run experiments to model preterm issues in humans. Preterm birth in humans can lead to impaired brain development and cell death, even without gross brain injury (Bennet, et al., 2013). Researchers assessing outcomes of traumatic brain injury in human children at several ages (in years 0-2, 3-6, 7-9, 10+) found the poorest outcomes were in the 7-9 year old group (e.g. Crowe, Catroppa, Babl, Rosenfeld, & Anderson, 2012). These human and rodent studies may point to similar mechanisms across these species following early and late juvenile injury.

Immune System Activation

The immune response is a powerful defense mechanism activated in response to some neural experiences. Astrocytes, glial cells within the brain, modulate a diverse range of plasticity

processes at the cellular level. Glial fibrillary acidic proteins (GFAP) are intermediate filament proteins which bind together to form astrocytes and Bergmann glia. GFAP and inflammation may be used as a marker for localized astrogliosis. Astrogliosis, which is an abnormal increase in the number of astrocytes, mediates inflammation in the extracellular environment, affecting neuronal survival and cognitive outcomes after brain injury (Sajja, Hlavac, &VandeVord, 2016). GFAP levels ares influenced by many experiences (e. g. drug addiction and learning), which in turn influences synaptic morphology (Scofield, et al., 2015). Experience-dependent GFAP alteration can disrupt or aid in neuronal excitability, synaptic transmission, and structure of neurons (Halassa, Fellin, & Haydon, 2007). Levels of astrogliosis vary alongside brain change, as the immune system is sensitive to both positive and negative experience.

Astrocytes are a necessary part of recovery and brain function, astrocytes limit damage with scar formation and post-insult remodeling. Astrocytes can prevent axonal regeneration and exacerbate inflammation via cytokine production (Sofroniew & Vinters, 2010). Sex differences of inflammatory responses may impact outcomes after brain trauma and psychological disorders as inflammation is involved in these circumstances. Astrogliosis occurs alongside many active processes involved in developmental plasticity. These processes are stimulated by both behaviour and neuronal activity. Brain development starts in the prenatal environment with neuronal proliferation and migration then continues with myelination into early adulthood. In humans, the brain continues to form synaptic connections after birth, begins myelinating axons, and then synaptic pruning refines connections to improve efficiency. Experience is a necessary and influential part of development in the mammalian brain. There are experience-expectant structures, which require specific types of experience to become functional. In order to better understand typical brain development, observation of the structural and biochemical changes that

occur in typical development and interrupted development reveals the strengths and weaknesses of age related plasticity. Sensory systems required specific sensory stimulation (e.g. visual) to develop typical cell maturation and connections. Another type of experience required for typical development gaining attention is ingestion of food and bacteria. The gut microbiome is linked to the nervous and immune system influencing whole body function. The infant gut requires specific nutritional and microbiota exposure for brain and immune system development, such as iron for hippocampal maturation (Beard, 2008) and microbiota for strong immune presence at the blood brain barrier (Al-Asmakh & Hedin, 2015). Development of the nervous and immune systems require several types of experience. Without necessary experiences, specific functions can fail to develop reducing the capacity of the individual.

The immune system responds to experience with microglia and inflammation, which can have both helpful and harmful effects in the brain. It may be that inflammation is only harmful when it is dysregulated by other factors, causing the naturally protective immune response to become maladaptive (Rivers-Auty & Ashton, 2014). In some cases of neuropathology (e.g. Parkinson's disease), microglia have a detrimental role and increase neurodegeneration (Joers, Tansey, Mulas, & Carta, 2016). Targeting immune processes to reduce microglia activity may aid in reversing or slowing maladaptive effects (Morara, Colangelo, & Provini, 2015). Inflammation involves both pro- and anti-inflammatory cytokines. Cytokines are a variety of small proteins which act on both proximal and distal cells. These cytokines are involved in multiple processes and serve different functions, for example, modulating pain, fever, and antibody production. Attempting to modulate inflammation may have effects on positive and necessary processes, as well as the problematic targets. Researchers are currently targeting cytokines to treat Alzheimer's disease, attempting to regulate neuroimmune function in

neuropathology to restore rather than destroy brain function. Investigating specific cytokines and other proteins aid in our understanding of adaptive and maladaptive immune functions.

Inflammation interacts with biological sex, producing distinct inflammatory fluctuations in males and females. The neuroimmune system has a central role in sexual differentiation of the brain and, as such, remain different, even in typical development, causing sex differences in number and morphology of astrocytes (Lenz & McCarthy, 2015). In pain circuitry, astrocytosis is more active in females than males (Mapplebeck, Beggs, & Salter, 2016). This sexual dimorphism is influenced by brain area. Exposure to dysregulation of the immune system in the prenatal environment causes an increase in GFAP in more brain areas in females compared to males (de Souza et al., 2015). This is evidence that sex differences are important to evaluate when considering brain change as results will vary based on sex. Studies have historically focused on males only, as such the conclusions may not be extended to females. Many conditions vary in both symptomology and recovery based on sex, such as stroke. After ischemic stroke sex-specific glia responses begin immediately, an increase in phagocytoic microglia was present in male, but not female mice (Morrison & Filosa, 2016). The immune system may be driving sex differences in many conditions. In stroke (Aquilani, et al., 2014), bipolar disorder (Muneer, 2016), and depression (Byrne, Whittle, & Allen, 2016), the effects of prolonged inflammation in the brain have been associated with maladaptive plastic change, inhibiting recovery. Sex differences in immune function must be considered in research design as sex linked immune differences may be displayed in various behavioural and biological tests.

Conclusion

Brain plasticity incorporates multiple overlapping experiences through development and into adulthood as structural and functional change. The early thoughts about the unchanging

nature of the mature brain needed to be repeatedly disproven in order for scientists to accept the changeable nature of the adult brain. Researchers with many different backgrounds (e.g. biology, kinesiology, and psychology) have different ideas and methodologies, which applied to studying the brain adds to the diversity of the research on brain plasticity. Yet, the relationship between brain change and inflammation is not well understood. Astrocytes are as ubiquitous as neurons, responsible for many mechanisms in maintenance and defense, as well as frequently influencing brain change. A complete understanding of brain change must include the effects and interactions of many concurrent mechanisms.

Chapter II. Brain Plasticity & Concussion

As efficient brain function is essential to long-term cognition and motor behaviour in children, incurring concussions or mild traumatic brain injuries (mTBIs) can be quite hazardous to brain processes and development. Concussion is a traumatic brain injury which occurs after a blow to the head or another part of the body caused the brain to move rapidly back and forth in the skull. This rapid movement causes a disturbance in brain function and several types of symptoms, including motor, memory, sleep, and emotional dysfunction. Children engage in a lot of adventurous play and, without a keen sense of balance, this can lead to a high risk of mTBI. Concussions have a highly variable range of symptoms with short- and long-term outcomes, both are difficult to predict.

Diagnosis

An important issue in concussion research is the lack of clarity in defining concussion. An injury is considered a concussion after a biomechanical force transmits to an individual's head which may or may not result in immediate clinical signs and symptoms. Most symptoms that develop are transient and clear up on their own, but researchers and clinicians are actively looking for novel ways to predict mTBI outcomes. Historically, brain imaging techniques have not been able to pick up the subtle disruption in the brain after concussion. One imaging technique, diffuse tensor imaging (DTI) highlights the space water can move through the brain, indicating where structure membranes are located and may reveal microstructural plastic change in the brain (Alexander, Lee, Lazar, & Field, 2007). DTI appears to detect concussion related brain change and could potentially be used to predict those at a greater risk of prolonged recovery (Murugavel et al., 2014). Attempts to use DTI to observe a patient's recovery have shown that this imaging technique may be used to determine the appropriate time to resume

normal activity levels (Farhadi & Lonser, 2013). The microstructural changes detected by DTI may also be indicative of anxiety or stress-based changes (Sekiguchi, et al., 2014), confounding any concussion-based change. Although this technique holds promise, physicians should be tentative to draw significant conclusions about these plastic changes indicated via DTI. Until better diagnostics and biomarkers are confirmed, we are left with a complex injury pattern and symptom-based assessments.

Children have difficulty communicating their symptoms after mTBI. Children's language and self-awareness is not fully developed making communication a potential obstacle for parents and physicians to understand the severity of child concussion. Pediatric concussion affects development with primary and secondary effects on plastic change. Primary effects are the first biological responses within the brain after injury, such as axonal damage. Secondary effects are numerous and more variable. There are internal secondary effects, which are slower reacting brain responses responding to any ongoing changes after mTBI that affect long-term outcome.

There are secondary effects related to a child's environmentally-induced brain changes in the time after experiencing a pediatric mTBI. After injury, children are instructed to rest from school and play, in order to allow the brain time to recovery without re-injury or aggravating symptoms (Giza & Kutcher, 2014). Time away from typical learning and social exposure may disrupt developmental processes, as these types of enrichment promotes brain plasticity. This time away from extra stimulation from the environment is thought to help brain recovery, however, reduced environmental enrichment may interact negatively with ongoing brain development, a time when experience is important for experience-dependent plasticity. There is also a period of vulnerability in the brain after concussion causing an increased risk of re-injury and a threat to long-term neurological function. Children are called "toddlers" at three years old

as they are uncoordinated and are still developing balance and motor skills. Underdeveloped motor skills can lead to increased falls and risk of concussion. There is a period of vulnerability in the brain after concussion causing an increased risk of re-injury and a threat to long-term neurological function (Eisenberg, Andrea, Meehan, & Mannix, 2013). Toddlers have a high risk of falling which can lead to mTBI. We do not know the risks of single and repeated mTBI in childhood and the potential interactions with development.

The restoration of brain function after concussion disrupts the brain's response to new and everyday experience. The disruption of typical neurological processes increases the likelihood of incurring a second mTBI, as cognitive and motor function may be impaired. A single mTBI can have a negative effect on future attentional and cognitive processes (Moore, et al., 2015). To avoid aggravating symptoms and re-injury, return-to-play protocols are recommended after incurring a concussion. These types of protocols recommend the patient rests, both physically and cognitively, to allow their brain the time it needs to recover. This period of rest is necessary as additional mTBIs cause a worsening and prolonging of symptoms (Terwilliger, Pratson, Vaughan, & Gioia, 2015). Although a period of rest is recommended for recovery from concussion, the mechanisms behind mTBI damage and recovery is unclear.

In an analysis of adolescent repeated concussion patients, researchers found symptoms worsened with both an increased number of past concussions and a decreased in time elapsed between concussion (Eisenberg, Andrea, Meehan, & Mannix, 2013). The restoration of brain function after concussion disrupts regular mechanisms in brain function, impacting visuomotor processing and balance. The disruption of typical neurological processes increases the likelihood of incurring a second mTBI, as cognitive and motor function may be impaired. A period of rest, both physically and cognitively, is necessary as additional mTBIs cause a compounding effect on

post-concussion symptoms (Terwilliger, Pratson, Vaughan, & Gioia, 2015). Even if a patient appears not to have any symptoms, their brain may still be recovering from the injury and a secondary impact could compound the deleterious effects. Premature activity and stimulation may harm patient recovery by interrupting the restful healing period, reducing the brain's healing ability, even without a second injury (Carson et al., 2014). The ongoing plastic changes causing diverse symptoms after concussion have not been clearly defined, as such it is difficult to aid recovery processes.

Although most concussion patients achieve neurological recovery within 1-2 weeks, some develop post-concussion syndrome, which can persist beyond one year. Post-concussion syndrome is a complex disorder with various persistent symptoms lasting at least three months after concussion. There are several factors that influence recovery after mTBI. Experience, such as a higher fat content in an individual's diet will cause epigenetic changes, increasing vulnerability to post-concussion syndrome (Myschasiuk, Hehar, Ma, & Esser, 2015). Biological sex is often used as a predictor for concussion outcomes. Female children and adolescents often have more symptoms (Dillard et al., 2016) and prolonged recovery (Miller et al., 2015) compared to male counterparts after concussion. This sex difference is not well understood. Sexual dismorphism of inflammation may be responsible for this difference, as inflammatory cascades are primary responders after mTBI (Briones, Woods, & Rogozinska, 2013). In pediatric mTBI, sex and developmental age influence the brain's inflammatory response to concussion and may factor in the development of post-concussion symptoms.

In order to determine more information about the ongoing plastic change after concussion, researchers are trying to find evidence of protein change in patients' blood suggesting changes in cellular functioning. Blood collected from patients can have varied levels of specific proteins,

pointing to plastic brain change after mTBI. One protein used as a marker of brain injury is S100B, which is released in the brain from astrocytes and readily passes through the blood brain barrier after traumatic brain injury (Kleindienst, Hesse, Bullock, & Buchfelder, 2007). S100B aids in neuronal survival during development and accelerates neuroinflammatory processes after hypoxic-ischemic injury (Wainwright et al., 2004). One problem with using S100B as a marker is the vulnerability to change due to non-concussion related factors. S100B can be influenced by patient age, previous injury status, and the time the blood was drawn (Filippidis, Papadopoulos, Kapsalaki, & Fountas, 2010). As many variables alter the levels of this biomarker, it difficult to draw definitive conclusions of plastic change, but S100B can be used to mark inflammation. Other proteins may be used as biomarkers in the future, making diagnosis easier and more definitive with regard to long-term outcomes.

Interacting Variables

The investigation of human mTBI is complicated by many confounding variables, including impact specific variables, including speed, point, and area of impact, as well as individual specific variables, such as age, sex, diet, immune function, and sleep patterns. Research with animal models occur in controlled environments, and give researchers the opportunity to make precise comparisons and examine brain tissue. In these controlled studies, animals will all have similar life experience, be at the same age, and receive the exact same injury, reducing interference from extraneous variables.

Interactions can be revealed with examination of sex, developmental age, and repeated injuries. By injuring animals at different ages internal and external measures can be compared to control animals. Previous research has looked at lesions and at various ages, but a concussion is significantly more mild than a lesion and the brain is better able to adapt to this type of injury.

Comparisons of several mTBI time points may determine which time points in development the brain's plasticity facilitates resilience or vulnerability after mild injury. A clearer picture of how the brain reacts to injury at several ages could help guide researchers of developmental brain trauma to provide individuals with specific treatment based on their age group.

mTBI Models

Methodology for modeling mTBI is diverse. Traumatic brain injuries may be classified as severe, moderate, or mild, where the mild type is often considered concussion, but there are not clear bounds on these levels. Modeling concussion in rodents has been done with different methods, with a range of precision and resulting trauma. Rodent models of pediatric mTBI have been used to investigate disruptions in developmental synaptic pruning (Mychasiuk, Hehar, Ma, Kolb, & Esser, 2015), deficits in motor coordination (Kane et al., 2011), abnormal social behaviour (Mychasiuk, Hehar, Farran, & Esser, 2014), and cortical thinning (Goddeyne, Nichols, Wu, & Anderson, 2015). Several studies involve opening the head to expose the skull before concussive impact (Dalgard, 2012; Briones, Woods, & Rogozinska, 2013). This method enables the researcher to direct the impact over a precise area of the brain. Despite the benefit of precision after exposing the skull, healing an open head wound requires a long recovery time, which causes a delay in behavioural research and potentially introduces confounds. Kane et al. (2011) did not use an invasive head-opening procedure (i.e. did not open the skull or scalp), but instead hit the anesthetized rodent with a guided weight almost as large as the animal's head. This procedure includes the rodent falling after the strike. This rotational fall is an important part of the injury, modeling the acceleration and deceleration of the brain in human injury (Kane et al., 2011). The benefits of both methods can be combined by using an mTBI procedure that employs a closed head method with a small impact probe. This procedure allows for fast

recovery time, the rotational fall, and a precise hit. Although there is a spectrum of injury severity in this type of research, each piece contributes to our understanding of injury related brain change, but not always in the area of concussion.

Conclusion

Children have a high risk of mTBI with a variable range of symptoms with unpredictable long-term outcomes. The lack of clarity in defining concussion leads to difficulty diagnosing and treating concussion. DTI and biomarkers are not developed enough to reliably indicate level of damage and recovery processes after concussion. Patient concussion history can used as a predictor for re-injury symptomology. Researchers and clinicians seeking new methods to monitor post-mTBI brain change to better predict outcomes and treat patients. Research with animal models of mTBI provides direct access to the brain and behaviour allowing for thorough analysis of outcomes. The combined work of physicians and researchers on human mTBI and rodent models is an ongoing effort towards a complete understanding of concussion.

Chapter III. Rodent Model of Repeated Pediatric Concussion

Throughout an animal's lifetime, experience continually shapes the brain chemically, morphologically, and functionally. Experience can vary from simple sensory input, thoughts and feelings, to mastering skills, trauma, and almost every type of experience. Neuroplasticity continually refines and shapes the brain, whether change is adaptive or not. A lot of plastic change happens around the synapse, but synaptic plasticity is not the only type of change. Brain plasticity can be assessed at many levels, from protein expression to behaviour. Although the brain is plastic at all ages, the most flexibility occurs during development, when it is more responsive to both positive and negative experiences. Children's developing brains have many active ongoing processes and similar experiences evoke different responses than in the adult brain. The process of maturation allows the brain to be hyperflexible. The increase of synapses and pruning that occur during development allow the brain to more readily respond to experience (Huttenlocher, 1979). It is not clear how childhood concussion, or mild Traumatic Brain Injury (mTBI), interact with development to modify the brain.

In order to model injury of the human toddler age, animals need to be at an age analogous based on brain development. Rat pups aged 21-24 days are at a similar developmental age to human children at 3-4 years old based on axonal, synaptic, and dendritic density, brain weight, myelination rate, and neurotransmitter changes, (as reviewed in Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). Structural and functional brain maturation within an animal model is important for comparison of human development and recovery.

The controlled lab setting for animal models of concussion reduce confounds of diverse experience. Modeling concussion in rodents has been done with different methods, with a range of precision and trauma in both open and closed head injuries. Open head injuries allow the

researcher to select brain area after opening the skull, but healing an open head wound requires a long recovery time, which causes a delay in behavioural research and introduces confounds. Kane et al. (2011) employed a general injury with a closed head and rotational fall. In order to best model human mTBI, our work (e.g. Dyck & Ivanco, 2018) includes a fall to provide acceleration and deceleration of the brain, with a closed head and a small impact probe for a precise hit over the right motor cortex.

The motor cortex is a flexible area of the brain, which can be more or less plastic based on age and sex (Polimanti, et al., 2016), indicating sensitivity in response to manipulation. We selected motor tasks to reveal differences in coordination, balance, and strength after injury to the motor cortex. A tapered beam can be used to assess limb coordination as rodents traverse the narrowing beam (Schellert, Woodlee, & Fleming, 2002). The hanging wire task determines forelimb strength and grasping ability, as pups attempt to hold onto a thin dowel to avoid falling (Rakhunde, Saher, & Ali, 2014). The rotarod evaluates motor performance using a rotating rod. This rod takes advantage of rats' natural fear of falling and duration rats remain balanced on the rod is recorded (Hamm, Pike, O'Dell, Lyeth, & Jenkins, 1994). Injuries over discrete brain areas may be most effectively tested with area specific tasks. With our focal injury we selected tasks to demonstrate dysfunction in the motor cortex.

In a previous study, we investigated a juvenile rat model of mTBI (Dyck & Ivanco, 2018). Using this model, we found an increase in Brain-Derived Neurotrophic Factor (BDNF), but no play behaviour deficits compared to sham animals, suggesting a long lasting molecular response. BDNF is a protein secreted in the brain related to experience driven plastic change, in this instance, as a recovery/compensation response. These results emphasize the underlying recovery and compensatory processes, and the ongoing plastic change without behavioural impairment

after an injury. Biological markers of recovery and compensation do not represent the level of damage after injury, but that the brain is working hard to adapt.

Biological markers of injury may reveal the level of damage after mTBI. One brain response to injury is inflammation, a defensive reaction that activates microglia to produce cytokines, chemokines, and growth factors to aid restoration (as reviewed in Jin & Yamashita, 2016). Inflammation has many roles within the brain, which are both positive and negative. In stroke (Aquilani, et al., 2014), bipolar disorder (Muneer, 2016), and depression (Byrne, Whittle, & Allen, 2016), the effects of inflammation in the brain have been associated with maladaptive plastic change, which are endogenous changes obstructing healthy function. Glial fibrillary acid proteins (GFAP) bind together to form astrocytes and have been used as a marker for localized astrocytosis (e.g. MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011), indicative of secondary neural damage (Lima et al., 2008). GFAP is been recognized as a marker of severity in moderate TBI using blood samples (Papa et al., 2016). Measuring levels of GFAP in brain tissue and blood identifies one aspect of the inflammatory response, specifically where astrocytes have increased. Levels of GFAP can be measured to reveal the total reactive astrocytosis as a regulator of the brain's plastic response to injury or infection.

A powerful modulator of brain plasticity and recovery from trauma is stress. Chronic and acute stress evoke sexual dimorphic neuroimmune mechanisms, causing altered microglial morphology and immune factor expression varying across brain region and sex (Bollinger, Collins, Patel, & Wellman, 2017). Endogenous glucocorticoids are measured as a marker of stress in mammals, and are released from the adrenal glands, interacting with the inflammatory system following an immune challenge (Bellavance & Rivest, 2014). Cortisol and corticosterone are the primary glucocorticoids produced in humans and rodents respectively. Cortisol and

corticosterone may be collected in saliva, hair/fur, and blood samples to reveal basic stress response in humans and rodents.

Here, we investigate behavioural and biological evidence of brain impairments with motor, balance, and strength tasks following single and repeated mTBI. We used a juvenile rat model of repeated mTBI to investigate dose-like effects of repeated mTBI. We tested the hypothesis that the mTBI rat pups would show graded deficits in behavioural performance and a graded increase in protein expression, increasing with the number of impacts. We also looked for a sex interaction with injury status, expecting greater behavioural deficits and higher levels in GFAP in injured females compared to injured males. Brain tissue was analyzed for GFAP and corticosterone.

Method

Animals

Our lab houses a breeding colony of Long-Evans rats, which provided and maintained the necessary animals for this project. Animals were kept in a temperature (22C +/-1) and humidity controlled (55% +/-3) room with a 12-hour light-dark cycle and access to food and water *ad libitum.* Animals were housed in standard shoebox cages with Sani-Chips bedding. Nylabones and red tubes were kept in cages as environmental enrichment. Animal behaviour was assessed immediately after sham/impact procedures, two hours after injury, and the following morning. Pups were weaned in the morning on post-natal day (PND) 21, then housed in groups of four littermates of the same sex. Each cage housed one animal from each group (sham, one, two, or three mTBIs). In each cage, animals were coded via different coloured tail rings denoting group identity. An equal number of both sexes (N = 64) were used in order to observe any sex differences. Pups were randomly assigned one of four conditions, incurring 1-3 impacts or the

sham procedure (see Table 1). Animals had a 24-hour recovery time separating each impact. This experiment was carried out in accordance with the Canadian Council of Animal Care guidelines and ethics was approved by the University of Manitoba Animal Care Committee.

Brain Injury Procedure

Materials. Animals were lightly anesthetized in a chamber with 3.5% isoflurane. The mTBIs were generated with a Leica Impact One (Leica Biosystems Richmond Inc.). This apparatus was attached to the ruled arms of a stereotaxic device (Narishige Scientific Instrument Laboratory) to ensure the animals were placed on the precisely measured foil. The 2mm impact probe was an instrument developed to strike rats and mice to model concussive-impact. The dwell timer determines the length of time the impact probe remains down. The probe was set to a brief 0.3 seconds as per the manufacturer's manual.

Procedure. The anesthetized animals were placed on foil attached to a modified stereotaxic frame under the impact probe. A new sheet of aluminum foil was measured, marked, and taped for each animal placement. The foil would be cut with a slit down the center and taped onto the arms of the stereotaxic device. A sponge was placed 12 cm below the foil to soften the animal's fall. Coordinates were measured and drawn on the foil ensuring each animal was accurately placed. The measurements indicated where the nose and ears line up to ensure the impact probe would hit the skull above the right motor cortex (9mm above and 2mm right of bregma). After the pup was briefly anesthetized and place on the foil, for the injury procedure the impact probe hit the animal, causing the foil to break and the animal to fall on a sponge below. As per lab protocol, the sham condition had all aspects of the injury procedure excluding the hit. The sham group animals were anesthetized, then placed on the foil and remained there when the probe

strikes high above the animal's head. Next, a slight pressure was applied to the foil causing the foil to break in order to simulate every aspect of the injury procedure without the impact.

To control for effects of handling and transportation, animals in each group were brought into the surgical suite on PND 21-23. All rats were kept in their home cage and placed on a table in the surgery room. The pups were marked with tail rings and handled. On PND 23, after injury/sham procedure, a second researcher recoded the animals' tail rings to ensure the primary researcher is blind to conditions during behavioural testing and analysis. Half of the sham group received anesthetic on each procedure day, the other half only received one dose of anesthetic. The group receiving three rounds of anesthesia was used to rule out effects of anesthesia.

Behaviour Tests

Animals ran on the tapered beam, wire hanging tasks, and rotarod. In order to observe how the motor cortex is affected after injury, relevant tasks were selected to test motor function, balance, and strength. Impacts to specific brain regions are best viewed through tasks designed to evaluate specific brain function. The tasks used do not require training and are simple enough for young animals. All rats were tested the morning following their first/final injury or sham procedure (PND 24). Each test required a maximum five minutes of active effort per animal. The animals had breaks in their home cage between each trial and task.

Tapered beam. The animals were habituated to this task the morning of the PND 23, which was either their first or final injury procedure/sham procedure. For this task, the animals traversed a 165 cm long tapered beam and time to cross the beam was recorded. The initial width of the beam was 6 cm, narrowing to 1.5 cm at the end (as per Schallert, et al., 2002). The home cage was placed at the end of the beam as an incentive to complete the task. Animals were scored on duration crossing the beam to reach the goal. Some animals were encouraged to

continue moving forward with a touch to the tail or side. The trials were video recorded to aid in offline scoring. The animals ran for three trials on the training and test day. Each rat had one minute of rest between trials.

Hanging wire. In this task, the animals were suspended from their forelimbs on a thin dowel (as per Rakhunde, et al., 2014). Thick foam was placed beneath the wire to ensure a soft landing for the pups. This task did not require habituation. Animals participated in two test trials. For this task latency to fall was recorded, time in seconds was scored. Trials were video recorded for precise time scoring. The animals were suspended for a maximum of 90 seconds and were removed at that point if they had not already dropped off.

Rotarod. The rod was set to 20 rotations per minute to test balance and time remaining on the rod was scored (Hamm, et al., 1994). This task did not require habituation. The rats were tested over three trials with a break between each trial. Duration of the time the rat remained on the rod was recorded. Trials were video taped in order to observe any variation in balance tactics. Thick foam was placed 30 cm below the rod to ensure gentle landing with each fall. There was a maximum time set at two minutes per trial, at which point rats would be removed from the apparatus. Only one trial reached this maximum.

Tissue Collection & Assays

Animals were euthanized 1-2 hours after the behavioural tasks were completed on PND 24. Rats were brought to a surgical plane in a chamber with 3.5% isoflurane, then maintained with a nose cone during saline perfusion. Blood was collected for analysis of levels of corticosterone. Blood was centrifuged fifteen minutes after collection and serum was stored in the -80C freezer until the time of analysis. Blood was analyzed for corticosterone levels with enzyme immunoassay (EIA) kits (DetectX, Arbor Assays, Ann Arbor, MI, USA) according to the

manufacturer's protocol. Tissue was extracted from the right motor cortex, the left motor cortex and the occipital lobe. Tissue was held in Eppendorf tubes in the -80C freezer until the time of analysis. These specific tissue selections were made based on vasculature and landmarks within the brain. Once all tissue was collected Bradford protein assays were used (as per lab protocol) to determine protein concentrations, we then equalized protein in our samples. Equal amounts of protein from all tissue was analyzed for GFAP with enzyme-linked immunosorbent assay (ELISA) kits (Promega Corpoation, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis

Behavioural data was analyzed with repeated-measures ANOVAs for each of the three tasks. Factors were defined as Sex (Male and Female), Impacts (Sham, One, Two, or Three impacts), and Trials for 2 X 4 X 2/3 analyses. Protein data was analyzed with 2 X 4 factorial ANOVAs. For GFAP levels, tissue from the right and left motor cortices were compared with the occipital lobe, 2x X 2y. An alpha of 0.05 was used to determine values that were statistically significant. Tukey's HSD were run to probe significant comparisons. Effect sizes were evaluated as small (0.01), medium (0.06), or large (0.14). Data was tested for outliers using a Grubb's test.

Results

Behavioural Results

The tapered beam did not reveal any significant differences in balance and there were no large effect sizes (see Figure 1). There was a main effect of injury for the wire hanging task, testing strength and grip maintenance, F (3,30) = 4.91, $p < 0.001$, $\eta = 0.21$. Sham and single-impact rats held on longer than double or triple impact rats (see Figure 2). Further probing

revealed the triple injury group dropped significantly quicker than the sham and single injury rats ($p = 0.01$, then $p = 0.02$, respectively). There was a main effect of sex for the rotarod, F (1,31) = 8.18, $p < 0.01$, $\eta = 0.13$. Female rats balanced on the rod longer than male rats (see Figure 3). Further probing did not reveal significant post-hoc comparisons.

Biological Results

There were two significant main effects in the corticosterone analysis. Corticosterone increased based number of impacts, F (3,93) = 3.01, $p = 0.036$, $\eta_p^2 = 0.125$. There was a significant sex difference in corticosterone expression, F (1,31) = 5.49, $p = 0.022$, $\eta_p^2 = 0.079$. Male rats had higher levels of corticosterone than females (see Figure 4). There were no main effects or interactions for GFAP protein level. There were two rats with a GFAP expression level higher than two standard deviations above the mean. We ran the analyses with the possible outliers in the two-impact group removed, but the results did not change and, therefore, we kept all data. The sham group had the lowest level of GFAP and the double injury group had the highest (see Figure 5).

Discussion

The data partially supported our hypothesis. Both sex and number of impacts influenced behavioural performance, GFAP expression, and corticosterone level. The wire hanging task revealed a dose-like effect of injury, the rats' duration holding on to the wire decreased after each concussion. The two-injury group expressed the highest level of both GFAP. Corticosterone level was significantly different for sex and number of impacts, without an interaction. The rotarod revealed sex differences, with the females remaining on the rod longer than males, but no effect of impact group. None of our analyses revealed an interaction of sex and injury status.

The male rats traversed the beam faster with increased number of impacts. We have reason to suspect this may be a result of impulse control, or a decreased fear response. Juvenile concussion can induce ADHD-like symptoms in children (Moore et al., 2015) and similar characteristics in rodent models (Hehar, Yeates, Kolb, Esser, & Mychasiuk, 2015). In rats, injured males are more likely to exhibit impulsive behaviour than sham animals (Hehar et al., 2015). In the present study, increased number of impacts in the male rat may have decreased impulse control causing increased speed on the tapered beam.

In both the sexes, as the number of impacts increased, the rats' ability to maintain balance on the dowel decreased in the hanging wire task. The experimental impacts on the right motor cortex may disrupt neuronal communication concerning static motor function, bimanual coordination, and/or neuromuscular control, affecting the rats' ability to hold themselves on the wire. Concussion causes microstructural changes to the corpus callosum (McAllister et al., 2012). The corpus callosum coordinates motor communication between the left and right hemispheres. After each impact, with our the corpus callosum may have incurred more damage causing the rats to perform worse on this task. The corpus callosum connects the left and right hemispheres. In our study we impacted the right motor cortex, but found similar amounts of GFAP in both the left and right motor cortices. As the corpus callosum is between the right and left motor cortices, it may have an influx of GFAP after impacts related to the motor deficits we have recorded.

The rotarod evaluates balance and coordination however, injury did not affect performance in this task. After injection of a neurotoxin long-term behavioural testing, including time to recover found mouse performance improved on the rotarod, but behaviour remained impaired on the wire hanging task (Huang et al., 2017). The wire hanging task may be more

sensitive than the rotarod in detecting subtle changes to motor function. The primary motor cortex is not solely responsible for motor function. The supplementary motor cortex, premotor cortex, and cerebellum have large roles in movement and coordination. In this study, the impact was directly over the right motor cortex, which likely caused the most dysfunction to the motor cortex, as well as disrupting other brain areas. The cerebellum, premotor and supplementary motor cortex may have functioned to compensate for the injured primary motor cortex.

The rotarod revealed a sex difference, as the females were able to stay on the rod longer than the males. Female rats are generally more agile than male rats, which may result in this sex difference. We were expecting an interaction between sex and concussion, which has been demonstrated in previous mTBI data with rodents (Mychasiuk, et al., 2014) and humans (Miller, et al., 2015). Our rats were tested on PND 24, which is the preadolescent stage in rats. There are fewer differences between males and females before adolescence. A sex and injury interaction may have been present if testing time was extended into adolescent development. In this study, we planned to observe changes immediately after injury.

The animals in the two-impact group had the highest level of GFAP expression in both motor cortices. There may be a maximum level of GFAP protein expression requiring a refractory period before levels may rise again. The three-impact group could have been in a refractory period, resulting in the two-impact group to have higher levels of GFAP. If we tested our rats after more recovery time there may have been more development of neuro-recovery mechanisms and changes in level of inflammation. Many studies collect tissue weeks post-mTBI (e.g. Mychasiuk, et al., 2014; Marco et al., 2013), but our study investigated a more immediate response. Our animals were culled at 24, 36, and 48 hours after their first injury. The three-impact group had more time to recover from their initial injury compared to the other groups.

Although all groups received their final injury on the same day, the difference of initial injury may have influenced the level of GFAP at the studies end point. Inflammatory cascades rise and fall in different stages of recovery depending on time and specific cytokine in question.

At toddler-like age, rodents have fewer sex differences than mature animals. The modest sex difference in juvenile rats may be why we did not find a sex and injury interaction in this study. If the rats were tested in adulthood, an injury and sex interaction may have developed in behaviour and/or brain function. Long-term outcomes are difficult to predict in immature brains, leading to various follow up questions with more recovery time.

The influence of isoflurane anesthesia is important to consider for interpreting our results. Anesthesia is necessary for reducing animal pain and stress during experimental procedures and tissue collection. In order to reveal the effects of various anesthetic one study compared brief exposure to isoflurane and CO_2 in adult rats and found typical corticosterone circadian rhythms were altered in the isoflurane group compared to the CO_2 group (Wren-Dail et al., 2017). This indicates that isoflurane alters corticosterone levels regardless of stress experience. In the present studies rats were exposed to isoflurane at the same time of day for all groups.

Cortisol in humans fluctuates in response to various experience throughout the day. The animals in the two-impact group had highest level of corticosterone. These animals also received a mid-range of anesthetic exposure time indicating the anesthetic was not the sole contributor to corticosterone change. Our rats were exposed to anesthesia for 2-4 consecutive days, the highest level at tissue collection. A comparison of experimental endpoint conducted with rapid decapitation and isoflurane anesthetic found isoflurane increased plasma corticosterone in female rats, but not males (Bekhbat, Merrill, Kelly, Lee, & Neigh, 2016). The exposure to isoflurane is an unavoidable drug for many studies, and may affect corticosterone in our study. That said,

animals were lightly anesthetized and recovered quickly. The isoflurane exposure was minimal, reducing the likelihood of an isoflurane-corticosterone interaction. As, such we feel confident that the outcomes reported here are a direct outcome of number of impacts and the brain response to the impacts, not any substantive role of minimal anesthesia.

Conclusion

We tested a juvenile rodent model of repeated concussion and found both sex and injury status influenced some behavioural performance and biomarkers. Our mild brain impact model produced bimanual coordination deficits in juvenile rats. Our findings suggest repetitive concussion generates early deficits in motor function, and an increase in corticosterone and GFAP in juvenile rats. Rats' ability to execute motor skills may be decreased with each injury. Concussions cause molecular cascades of both injury and recovery responses which are complicated by age and number of injuries. This study contributes to our understanding of brain change after single and repeated pediatric concussions, and suggests an additive effect to both behavior and brain measures. Our data supports the suggestion that multiple impacts can be far more serious than single impacts.

Chapter V. Future Directions

This research set out to investigate behavioural and biological changes after repeated pediatric concussion. Concussions during childhood can interrupt typical brain development (Carson, et al., 2014). The purpose of this study was to investigate protein and hormone changes after repeated pediatric concussion recovery and how behaviour is affected during recovery. We found repeated concussion caused some graded behaviour deficits, an increase in neuroinflammation, and increase corticosterone protein levels in juvenile rats. There was no sex-injury interaction for any of the behaviour tasks or protein levels. In our study, there may have been a maximum response of GFAP after two successive injuries causing immune exhaustion. Another possibility is post-concussion neuroinflammation may have varied based on time allowed for repair mechanisms to become fully active. Concussion-induced changes may have long term impact on development.

Moving forward with our repeated mpTBI model, there are many aspects of brain plasticity that could be further investigated. With the opportunity to carry out more projects with this repeated mpTBI, model I would compare more behavioural and protein outcomes with multiple time points in order to enhance our understanding of concussion outcomes. Introducing several cull points may reveal the development of recovery mechanisms over several stages of recovery. If we set end points at twenty-four hours, one week, and two months we could compare injured rat brains throughout recovery. Several end points would allow for observation of any injury related developmental changes. Our long-term group could complete multiple tasks evaluating learning and anxious/depressive behaviour throughout their recovery. If animals were tested for anxious/depressive behaviour the findings may corroborate corticosterone results. Tissue analysis from multiple cull points may establish the development and peak of the inflammatory

cascade. Comparing single and repeated concussion over time will reveal recovery differences after repeated hits. Studying all timelines and number of impacts was clearly beyond the scope of this project.

Developing brains have many active ongoing processes and similar experiences evoke different responses than in the adult brain. The process of maturation allows the brain to be hyperflexible. Comparing animals injured at different stages of development may reveal periods of resilience and vulnerability. With animals hit at different ages we could compare biological and behavioural recovery to further our understanding how concussion disrupts development.

Collecting tissue from additional areas would increase outcome measures possible with this experiment. I would extend collection from the impacted and contralateral areas to include the corpus callosum and hippocampus. Observing similarities and differences between impacted brain area and areas typically responsive to injury would contribute to our knowledge on post-injury plasticity in various areas. Collecting corpus callosum and hippocampus would assist in comparing our focal mTBI model with typical diffuse mTBI models. The both the hippocampus (Kane et al., 2011) and corpus callosum (Murugavel et al., 2014) have been shown to react with an increase of inflammation after concussion. Complete comparisons of mTBI models may expose substantial differences in recovery, aiding in our understanding of concussion outcome divergence.

Protein analysis assays are very costly. I was awarded a University of Manioba's J.G. Fletcher Award for my Master's proposal. This award contributed $3000 toward research expenses, but this did not cover the complete cost of the four GFAP ELISA kits. Although my work has experimental value with one assay, we had considered running more assays. More kits, however, were not in our budget. Ideally, we would have run an analysis phosphorylated tau (p-

Tau). Tau proteins help stabilize axons and aid in their flexibility in forming new synaptic connections. Increased p-Tau is correlated with cognitive impairment and brain network dysfunction, observable through neuroimaging with magnetoencephalography (Canuet, et al., 2015). The level of p-Tau increases during periods of increased plasticity such as early development (Brion, Octave, & Couck, 1994). Hyperphosphorylation of Tau protein may start immediately after injury, then spread throughout the brain over the restoration period. p-Tau may be related to the immediate cognitive and motor deficits after concussion, as it is increased during cognitive impairment and after injury (Hu, Tung, Zhang, Liu, & Iqbal, 2018). Accumulation of p-Tau is a part of typical aging, but, in cases of highly repetitive mTBI, p-Tau is distinctly built up in both neurons and glia cells, similar to patients with Alzheimer's disease (Hay, Johnson, Smith, & Steward, 2016). Long-term dysregulation of p-Tau may contribute to severe cognitive, emotive, and memory impairment in a broad variety of pathologies.

A helpful addition to our design would be measuring the duration of isoflurane exposure and attempt to ensure exposure is equal across all animals. Research indicating the stress-isoflurane interaction is novel and sufficient alternatives have not been developed for our methods. Reactions to isoflurane vary for each animal. For our purposes, the animal was anesthetized as briefly as possible to reduce isoflurane interactions. Attempting to control isoflurane level for equal exposure across groups is the best procedure at this time. In order to control the time animals are exposed to isoflurane each animal should be in the chamber after levels have stabilized and for the same amount of time. Recording the time for the animal to completely stop moving and time to right (after the animal to wakes and can flip from on their back to their feet) after waking may be useful in monitoring anesthetic effects.

Rodent models of concussion often use time to right as an indicator of concussion compared to the sham models. If we collected time to right data, we still may not find degrees of concussion symptomology. In our design time to right may not be possible as our animals are only briefly sedated and sometimes are awake immediately after falling. Time to right is easier to record in animals after they have been fully sedated. It is worth analyzing in an attempt to find any slight differences in immediate motor function in sham and injury groups. If there were not time to right differences by injury group then it would be only an effect of the isoflurane, in which case we could compare time to sleep with time to right and find individual responses.

Of our three selected motor tasks, the wire hanging task was the only test that revealed an impact dosage effect. Repeated test days with the tapered beam and rotarod may have revealed significant differences in these tasks. The uninjured animals may have mastered the tasks more quickly than the impacted animals after more experience with the tasks. We wanted to find immediate behaviour differences, testing on multiple days was not an option. If our animals would have been trained more before the test day, more practice may have revealed deficits in the impacted animals compared to the shams.

It was difficult to select motor tasks that were simple enough for young animals to perform, as young rat pups are not physically and mentally capable of completing many of the behavioural tasks for adult rats. Learning tasks are too difficult for young pups, as they cannot meet the demands of complex tasks. Juveniles can complete tasks testing anxiolytic behaviour (e.g. elevated plus maze and the light dark box). Emotional well-being tasks could be tested with a young rat cohort, which may increase our understanding of emotional changes after pediatric mTBI.

Altogether, the proposed additions to our original design would enhance our understanding of both biological and behaviour changes after repeated pediatric concussion. Further studies with our mTBI model would allow for more comparisons with a range of mTBI models with various levels of injury and would help to draw parallels with human concussion. Concussion awareness is on the rise, but we do not fully understand the neural mechanisms behind concussion. Collectively, integrated mTBI research will reveal the brain's recovery mechanisms and aid in assessment and treatment of childhood concussion. Broadly translated, our findings indicate that pediatric concussion causes motor dysfunction, specifically bimanual coordination deficits, and an increase in stress hormones and inflammation in the brain. The data presented in this thesis adds to the foundation of this specific repeated mTBI model. These results contribute to the literature on post-concussion rodent brain and behaviour change.

References

Al-Asmakh, M. & Hedin, L. (2015). Microbiota and the control of the blood tissue barriers. *Tissue Barriers, 3*(3), e1039691. doi: 10.1080/21688370.2015.1039691

Alexandar, A. L., Lee, E. L., Lazar, M., & Field, A. S. (2007). Diffusion tensor imaging of the brain. *Neurotherapeutics, 4*(3), 316-329. doi: 10.1016/j.nurt.2007.05.011

Aquilani, R., Boselli, M., Paola, B., Pasini, E., Iadarola, P., Verri M., … Boshi, F. (2014). Is stroke rehabilitation a metabolic problem? *Brain Injury, 28*(2), 161-73. doi: 10.3109/02699052.2013.860470

Beard, J. L. (2008). Why iron deficiency is important in infant development. *Journal of Nutrition, 138*(12), 2534-2536. doi: 10.1016/j.jpeds.2006.06.065

Bekhbat, M., Merrill, L., Kelly S. D., Lee, V. K., & Neigh G. N. (2016). Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: Implications for tissue collection methods. *Behavioural Brain Research, 305*, 122-25. doi: 10.1016/j.bbr.2016.03.003

Bellavance, M.-A., & Rivest, S. (2014). The HPA – Immune Axis and the Immunomodulatory Actions of Glucocorticoids in the Brain. *Frontiers in Immunology*, *5*, 136. http://doi.org.uml.idm.oclc.org/10.3389/fimmu.2014.00136

Bennett, E. L., Diamond, M. C., Krech, D., & Rosenzweig, M. R. (1964). Chemical and anatomical plasticity of brain. *The Journal of Neuropsychiatry and Clinical Neurosciences, 8*(4), 459-470. doi: 10.1126/science.146.3644.610

Bollinger, J. L., Collins, K. E., Patel, R., & Wellman, C.L. (2017). Behavioral stress alters corticolimbic microglia in a sex- and brain region-specific manner. *Plos One, 12*(12): e0187631. doi: 10.1371/journal.pone.0187631

Briones L. T., Woods, J., & Rogozinska M. (2013). Decreased neuroinflammation and increased brain energy homeostasis following environmental enrichment after mild traumatic brain injury is associated with improvement in cognitive function. *Acta Neuropathologica Communications, 1*(57), doi:10.1186/2051-5960-1-57

Brion, J. P., Octave, J. N., & Couck, A. M. (1994). Distribution of the phosphorylated microtubule-associated protein tau in developing cortical neurons. *Neuroscience. 63*(3):895-909. doi: 10.1016/0306-4522(94)90533-9

Byrne, M. L., Whittle, S., & Allen, N. B. (2016). The Role of Brain Structure and Function in the Association Between Inflammation and Depressive Symptoms: A Systematic Review. *Psychosomatic Medicine*. doi: 10.1097/PSY.0000000000000311

Carson, J. D., Lawrence, D. W., Kraft, S. A., Garel, A., Snow, C. L., Chatterjee, A., ... & Frémont, P. (2014). Premature return to play and return to learn after a sport-related concussion Physician's chart review. *Canadian Family Physician*,*60*(6), e310-e315.

Crichton-Browne, J. (1879). On the weight of the brain and its component parts in the insane. *Brain, a Journal of Neurology,* 42-67. doi: 10.1093/brain/2.1.42

Cynader, M., Berman, N., & Hein, A. (1976). Recovery of function in cat visual cortex following prolonged deprivation. Experimental Brain Research, 25(2), 139-56.

Dalgard, C. L., Cole, J. T., Kean, W. S., Lucky, J. J., Sukumar, G., McMullen, D. C., . . . Watson, W. D. (2012). The cytokine temporal profile in rat cortex after controlled cortical impact. *Frontiers in Molecular Neuroscience, 5,* 1-10. doi: 10.3389/fnmol.2012.00006

De Souza, D. F., Wartchow, K. M., Lunardi, P. S., Brolese, G., Tortorelli, L. S., Batassini, C., … Gonçalves, C. A. (2015). Changes in astroglial markers in a maternal immune activation

model of schizophrenia in wistar rats are dependent on sex. *Frontiers in Cellular Neuroscience,* 9:489. doi: 10.3389/fncel.2015.00489

Dillard, C., Ditchman, N., Nersessova, K., Foster, N., Wehman, P., West, P., … Julie Neblett, J. (2016): Post-concussion symptoms in mild traumatic brain injury: findings from a paediatric outpatient clinic, *Disability and Rehabilitation,* 1-7. doi:10.3109/09638288.2016.1152602

Dougan, B. K., Horswill, M. S., & Geffen, G. M. (2014). Athletes' age, sex, and years of education moderate the acute neuropsychological impact of sports-related concussion: A meta-analysis. *Journal of the International Neuropsychological Society, 20*(1), 64-80. doi:10.1017/S1355617712001464

Dyck, A. C. F. & Ivanco T. L. (2018) BDNF expression increases without changes in play behavior following concussion in juvenile rats (Rattus Norvegicus): brief report, *Developmental Neurorehabilitation,* doi: 10.1080/17518423.2018.1460878

Eisenberg, M. A., Andrea, J., Meehan, W., & Mannix, R. (2013). Time interval between concussions and symptom duration. *Pediatrics,* 132(1), 8-17.

Farhadi, H. F. & Lonser, R. R. (2013). Diffusion tensor imaging in the spotlight on concussion. *World Neurosurgery, 80*(6), 794-795. doi: 10.1016/j.wneu.2013.10.023

Filippidis, A. S., Papadopoulos, D. C., Kapsalaki, E. Z., & Fountas, K. N. (2010). Role of the S100B serum biomarker in the treatment of children suffering from mild traumatic brain injury. *Neurosurgical Focus, 29*(5), E2. doi: 10.3171/2010.8.FOCUS10185.

Flexner, L. B., Flexner, J. B., & Roberts, R. B. (1967). Memory in mice analyzed with antibiotics. Antibiotics are useful to study stages of memory and to indicate molecular events which sustain memory. *Science, 155*(3768), 1377-1383

Giza, C. C., & Kutcher, J. S. (2014). An introduction to sports concussions. *CONTINUUM: Lifelong Learning in Neurology, 20(6)*, 1545-1551. doi:http://dx.doi.org.uml.idm.oclc.org/10.1542/peds.2013-0432

Goddeyne, C., Nichols, J., Wu, C., & Anderson, T. (2015). Repetitive mild traumatic brain injury induces ventriculomegaly and cortical thinning in juvenile rats. *Journal of Neurophysiology, 113*(9), 3268-3280. doi:http://dx.doi.org.uml.idm.oclc.org/10.1152/jn.00970.2014

Halassa, M. M., Fellin, T., & Haydon, P. G. (2007). The tripartite synapse: roles for gliotransmission in health and disease. *Molecular Medicine, 13*(2), 54-63. doi:10.1016/j.molmed.2006.12.005

Hamm, R. J., Pike, B. R., O'Dell, D. M., Lyeth, B. G., & Jenkins, L. W. (1994). The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. *Journal of Neurotrauma, 11*(2), 187-196.

Hay, J., Johnson, V. E., Smith, D. H., & Steward, W. (2016). Chronic Traumatic Encephalopathy: The Neuropathological Legacy of Traumatic Brain Injury. *Annual Review of Pathology, 11*, 21-45. doi:10.1146/annurev-pathol-012615-044116

Hehar H., Yeates K., Kolb B., Esser M. J., Mychasiuk, R. (2015) Impulsivity and Concussion in Juvenile Rats: Examining Molecular and Structural Aspects of the Frontostriatal Pathway. PLOS ONE 10(10): e0139842. doi:10.1371/journal.pone.0139842

Herre, W. (1958). Environmental influences on the mammalian brain. *Deutsche Medizinische Wochenschrift, 83*(36), 1568-1574.

Holsapple, M. P., West, L. J., & Landreth, K. S. (2003). Species comparison of anatomical and functional immune system development. *Birth Defects Research Part B, 68*(4), 321-334. doi: 10.1002/bdrb.10035

Hu, W., Tung, Y. C., Zhang, Y., Liu, F., & Iqbal, K. (2018). Involvement of activation of asparaginyl endopeptidase in tau hyperphosphorylation in repetitive mild traumatic brain injury. *Journal of Alzheimer's Disease*, 1-14. doi: 10.3233/JAD-180177

Huang, D., Wang, Z., Tong, J., Wang, M., Wang, J., Xu, J. … Huang F., (2017) Long-term changes in the nigrostriatal pathway in the MPTP mouse model of parkinson's disease. *Neuroscience, 369*, 303-313. doi: 10.1016/j.neuroscience.2017.11.041

Jin, X. & Yamashita, T. (2016). Microglia in central nervous system repair after injury. *Journal of Biochemistry, 8*. doi: 10.1093/jb/mvw009

Joers, V., Tansey, M. G., Mulas, G., & Carta, A. R. (2016). Microglial phenotypes in Parkinson's disease and animal models of the disease. *Progress in Neurobiology.* doi:10.1016/j.pneurobio.2016.04.006

Kane, M.J., Angoa-Pérez, M., Briggs, D.I., Viano, D.C., Kreipke, C.W., & Kuhn, D.M. (2011) A mouse model of human repetitive mild traumatic brain injury. *Neuroscience Methods*. 203, (1), 41-49. doi: 10.1016/j.jneumeth.2011.09.003

Keating, M. J. & Gaze, R. M. (1970). Rigidity and plasticity in the amphibian visual system. *Brain, Behavior, and Evolution, 3,* 102-120. doi: 10.1159/000125465

Kleindienst, A., Hesse, F., Bullock, M. R., Buchfelder, M. (2007). The neurotrophic protein S100B: value as a marker of brain damage and possible therapeutic implications. *Progress in Brain Research, 161*, 317-325. doi:10.1016/S0079-6123(06)61022-4

Kolb, B. (1987). Recovery from early cortical damage in rats: I. differential behavioral and anatomical effects of frontal lesions at different ages of neural maturation. *Behavioural Brain Research, 25*(3), 205-220.

Kolb B, Petrie B, & Cioe J. (1996). Recovery from early cortical damage in rats, VII. Comparison of the behavioural and anatomical effects of medial prefrontal lesions at different ages of neural maturation. *Behavioural Brain Research, 79*(1-2) 1-13. doi:10.1016/0166-4328(95)00254-5

Kolb, L. C. (1949). An evaluation of lobotomy and its potentialities for future research in psychiatry and the basic sciences. *Journal of Nervous and Mental Disease, 110*, 112-148. doi: 10.1097/00005053-194911020-00002

Krech, D., Rosenzweig, M. R., & Bennett, E. L. (1960). Effects of environmental complexity and training on brain chemistry. *Journal of Comparative and Physiological Psychology, 53*(6), 509-519. doi:http://dx.doi.org/10.1037/h0045402

Lima, R., Guimaraes-Silva, J., Oliveira, J., Costa, A., Souza-Rodrigues, R., Dos Santos, C., … Gomes-Leal, M. (2008). Diffuse axonal damage, myelin impairment, astrocytosis and inflammatory response following microinjections of NMDA into the rat striatum. *Inflammation, 31*(1), 24-35. doi: 10.1007/s10753-007-9046-y

Lenz, K. M., & McCarthy, M. M. (2015). A starring role for microglia in brain sex differences. *The Neuroscientist,21*(3), 306-321. doi: 10.1177/1073858414536468

MacFabe, D. F., Cain, N. E., Boon, F., Ossenkopp, K., & Cain, D. P. (2011). Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. *Behavioural Brain Research, 217*(1), 47-54 doi:http://dx.doi.org.uml.idm.oclc.org/10.1016/j.bbr.2010.10.005

Mapplebeck, J. C. S., Beggs, S., & Salter M. W. (2016). Sex differences in pain: a tale of two immune cells. *Pain, 156*, 2-6. doi: 10.1097/j.pain.0000000000000389

Marco, E. M., Valero, M., de, l. S., Aisa, B., Borcel, E., Ramirez, M. J., & Viveros, M. (2013). Maternal deprivation effects on brain plasticity and recognition memory in adolescent male and female rats. *Neuropharmacology, 68*, 223-231. doi:10.1016/j.neuropharm.2012.08.014

McAllister, T. W., Ford, J. C., Songbai, J. I., Beckwith, J. G., Flashman, L. A., Paulsen, K., & Greenwald, R. M. (2012) Maximum principal strain and strain rate associated with concussion diagnosis correlates with changes in corpus callosum white matter indices. *Annals of Biomedical Engineering, 40*(1), 127-140. doi: 10.1007/s10439-011-0402-6

Miles, F. A. & Fuller, J. H. (1974). Adaptive plasticity in the vestibulo-ocular responses of the rhesus monkey. *Brain Research, 80,* 512-516. doi: 10.1016/0006-8993(74)91035-X

Miller, J. H., Gill, C., Kuhn, E. N., Rocque, B. G., Menendez, J. Y., O'Neill, J.A., … Johnston, J.M. (2015). Predictors of delayed recovery following pediatric sports-related concussion: a case-control study. *Journal of Neurosurgery Pediatrics.* 1-6. doi:10.3171/2015.8.PEDS14332

Moore, D. R., Pindus, D. M., Raine, L. B., Drollette, E. S., Scudder, M. R., Ellemberg, D., & Hillman, C. H. (2015). The persistent influence of concussion on attention, executive control and neuroelectric function in preadolescent children. *International Journal of Psychophysiology, 99,* 85-95 doi:\10.1016/j.ijpsycho.2015.11.010

Morara, S., Colangelo, A. M., & Provini, L. (2015). Microglia-Induced Maladaptive Plasticity Can Be Modulated by Neuropeptides In Vivo. *Neural Plasticity*, *2015*, 135342. doi:10.1155/2015/135342

Morrison, H. W., & Filosa, J. A. (2016). Sex differences in astrocyte and microglia responses immediately following middle cerebral artery occlusion in adult mice. *Neuroscience, 339,* 85–99. doi:10.1016/j.neuroscience.2016.09.047

Muneer, A. (2016). Bipolar Disorder: Role of Inflammation and the Development of Disease Biomarkers. *Psychiatry Investigation, 13*(1), 18-33. doi: 10.4306/pi.2016.13.1.18

Murugavel, M., Cubon, V., Putukian, M., Echemendia, R., Cabrera, J., Osherson, D., & Dettwiler, A. (2014). A longitudinal diffusion tensor imaging study assessing white matter fiber tracts after sports-related concussion. *Journal of Neurotrauma, 31*(22), 1860-1871. doi:/10.1089/neu.2014.3368

Mychasiuk R., Hehar H., Farran A., & Esser M. J. (2014). Mean girls: Sex differences in the effects of mild traumatic brain injury on the social dynamics of juvenile rat play behaviour. *Behavioural Brain Research,* 259, 284–291. doi:10.1016/j.bbr.2013.10.048

Mychasiuk, R., Hehar, H., Ma, I., Kolb, B., & Esser, M.J. (2015). The development of lasting impairments: A mild pediatric brain injury alters gene expression, dendritic morphology, and synaptic connectivity in the prefrontal cortex of rats. *Neuroscience, 288,* 145-155. doi: 10.1016/j.neuroscience.2014.12.034

Papa, L., Brophy, G. M., Welch, R. D., Lewis, L. M., Braga, C. F., Tan, C. N., … Hack D. C., (2016). Time course and diagnostic accuracy of glial and neuronal blood biomarkers GFAP and UCH-L1 in a large cohort of trauma patients with and without mild traumatic brain injury. *JAMA Neurology*. doi:10.1001/jamaneurol.2016.0039.

Polimanti, R., Simonelli, I., Zappasodi, F., Ventriglia, M., Pellicciari, M. C., Benussi, L., . . . Tecchio, F. (2016). Biological factors and age-dependence of primary motor cortex experimental plasticity. *Neurological Sciences, 37*(2), 211-218. doi:10.1007/s10072-015-2388-6

Rakhunde, P. B., Saher, S., & Ali, S. A. (2014). Neuroprotective effect of Feronia limonia on ischemia reperfusion induced brain injury in rats. *Indian Journal of Pharmacology*, *46*(6), 617–621. doi:10.4103/0253-7613.144920

Raisman, G. & Field P. M. (1973) A quantitative investigation of the development of collateral reinnervation after partial deafferentation of the septal nuclei. *Brain Research, 50*(2), 241-264. doi:10.1016/0006-8993(73)90729-4

Riesen, A. H. & Aarons, L. (1959). Visual movement and intensity discrimination in cats after early deprivation of pattern vision. *Journal of Comparative and Physiological Psychology, 52*(2), 142-149.

Rivers-Auty, J., & Ashton, J. C. (2014). Neuroinflammation in ischemic brain injury as an adaptive process. *Medical Hypotheses, 82*(2), 151-158. doi:10.1016/j.mehy.2013.11.024

Schallert, T., Woodlee, M. T., & Fleming, S. M. (2002). Disentangling multiple types of recovery from brain injury. *Pharmacology Cerebral Ischaemia.* 201–216

Schrader P. J. & Robinson, M. F. (1945). An evaluation of prefrontal lobotomy through ward behavior. *Journal of Abnormal and Social Psychology,* 401, 61-69. doi: 10.1037/h0054461

Scofield, M., Li, H., Siemsen, B. M., Healey, K. L., Tran, P. K., Woronoff, N., Boger, H. A., … Reissner, K. J. (2015). Cocaine self-administration and extinction leads to reduced glial fibrillary acidic protein expression and morphometric features of astrocytes in the nucleus accumbens core. *Biological Psychiatry*, doi: 10.1016/j.biopsych.2015.12.022

Segerstrom, S. C., Sephton, S. E., & Westgate, P. M. (2017). Intraindividual variability in cortisol: Approaches, illustrations, and recommendations, *Psychoneuroendocrinology, 78*, 114-124, doi: 10.1016/j.psyneuen.2017.01.026

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology, 106-107*, 1-16. doi:10.1016/j.pneurobio.2013.04.001

Sekiguchi, A., Sugiura, M., Taki, Y., Kotozaki, Y., Nouchi, R., Takeuchi, H., … Kawashima, R. (2014). White Matter Microstructural Changes as Vulnerability Factors and Acquired Signs of Post-Earthquake Distress. *PLoS ONE, 9*(1), e83967. doi: 10.1371/journal.pone.0083967

Terwilliger, V. K., Pratson, L., Vaughan, C, G., & Gioia, G. (2015). Additional Post-Concussion Impact Exposure May Affect Recovery in Adolescent Athletes. *Journal of Neurotrauma* doi:10.1089/neu.2015.4082

Wainwright, M. S., Craft, J. M., Griffin, S. T., Marks, A., Pineda, J., Padgett, K. R., & Van Eldik, L. J. (2004) Increased susceptibility of S100B transgenic mice to perinatal hypoxia-ischemia. *Annals of Neurology, 56*(1), 61-7. doi: 10.1002/ana.20142

Westfall, D. R., West, J. D., Bailey, J. N., Arnold, T. W., Kersey, P. A., Saykin, A. J., & McDonald B. C. (2015). Increased brain activation during working memory processing after pediatric mild traumatic brain injury (mTBI). *Journal of Pediatric Rehabilitation Medicine, 8*(4), 297-308. doi: 10.3233/PRM-150348

Wren-Dail, M. A., Dauchy, R. T., Blask, D. E., Hill, S. M., Ooms, T. G., Dupepe, L. M., & Bohm, R. P. (2017). Effect of Isoflurane Anesthesia on Circadian Metabolism and Physiology in Rats. *Comparative Medicine*, *67*(2), 138–146. PMCID: PMC5402733

Zhang, L., Li, X. X., & Hu, X. Z. (2016). Post-traumatic stress disorder risk and brain-derived neurotrophic factor Val66Met. *World Journal of Psychiatry, 6*(1), 1-6. doi: 10.5498/wjp.v6.i1.1

Zemek, R., Barrowman, N., Freedman, S. B., Gravel, J., Gagnon I., McGahern, C., … Osmond, M. H. (2016). Clinical risk score for persistent postconcussion symptoms among children with zcute concussion in the ED. *JAMA, 315*(10), 1014-1025. doi:10.1001/jama.2016.1203

Appendix

Table 1

Animal Group	**Impact/Anesthetic Days**	**Number of Animals**
Sham	Post Natal Day 23	16 (8 Male + 8 Female)
1 Impact	PND 23	16 (8 Male + 8 Female)
2 Impacts	PND 22 + 23	16 (8 Male + 8 Female)
3 Impacts	PND 21 + 22 + 23	16 (8 Male + 8 Female)
Total		64 (32 Male + 32 Female)

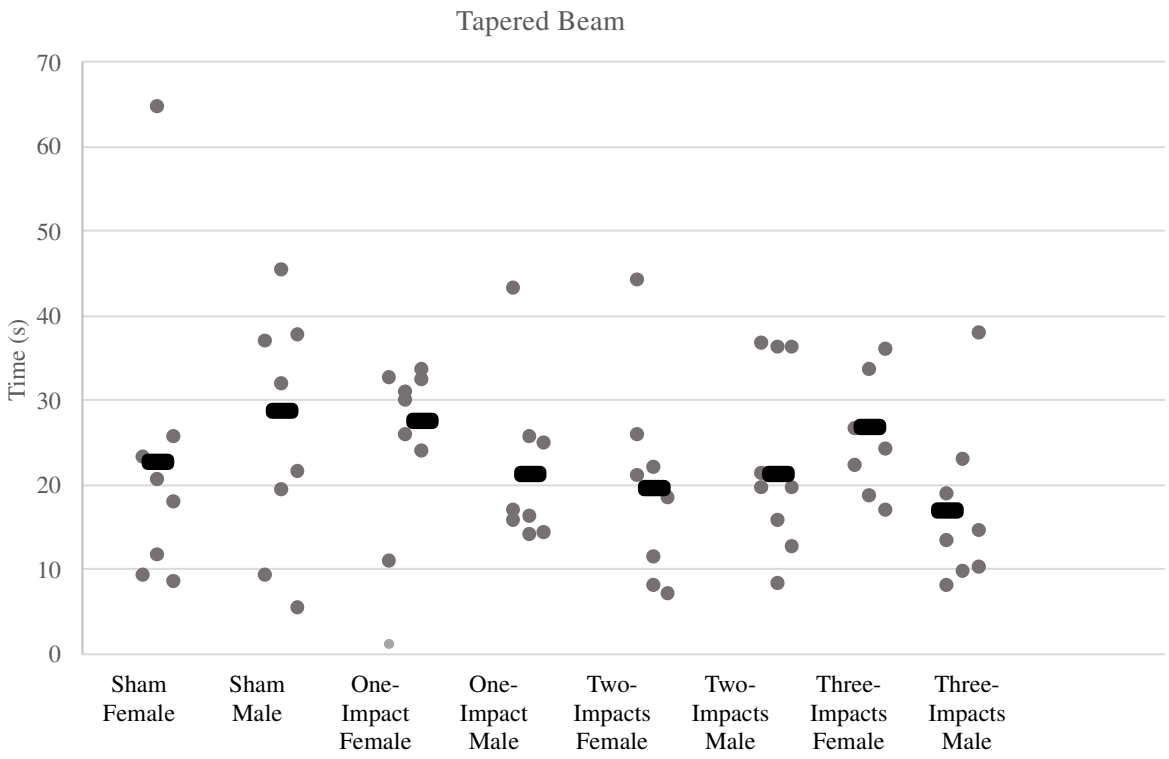

Figure 1. Time in seconds to traverse the tapered beam. Individual animal scores are the grey dots and group means are black lines. There we no significant differences found in this task.

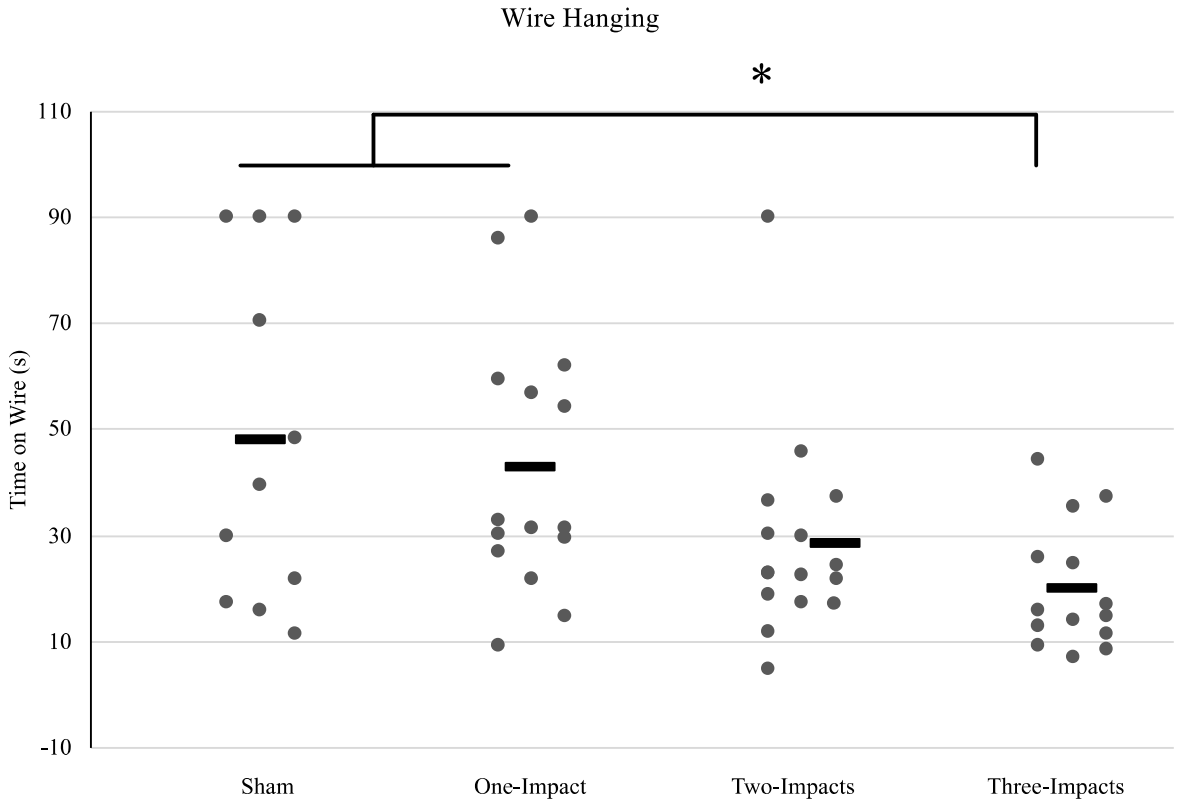

Figure 2. Time in seconds pups held onto the wire in the wire hanging task. Individual animal scores are the grey dots and group means are black lines. The three-impact group remained on the wire significantly less than the sham and single-impact groups (* indicates $p < 0.05$).

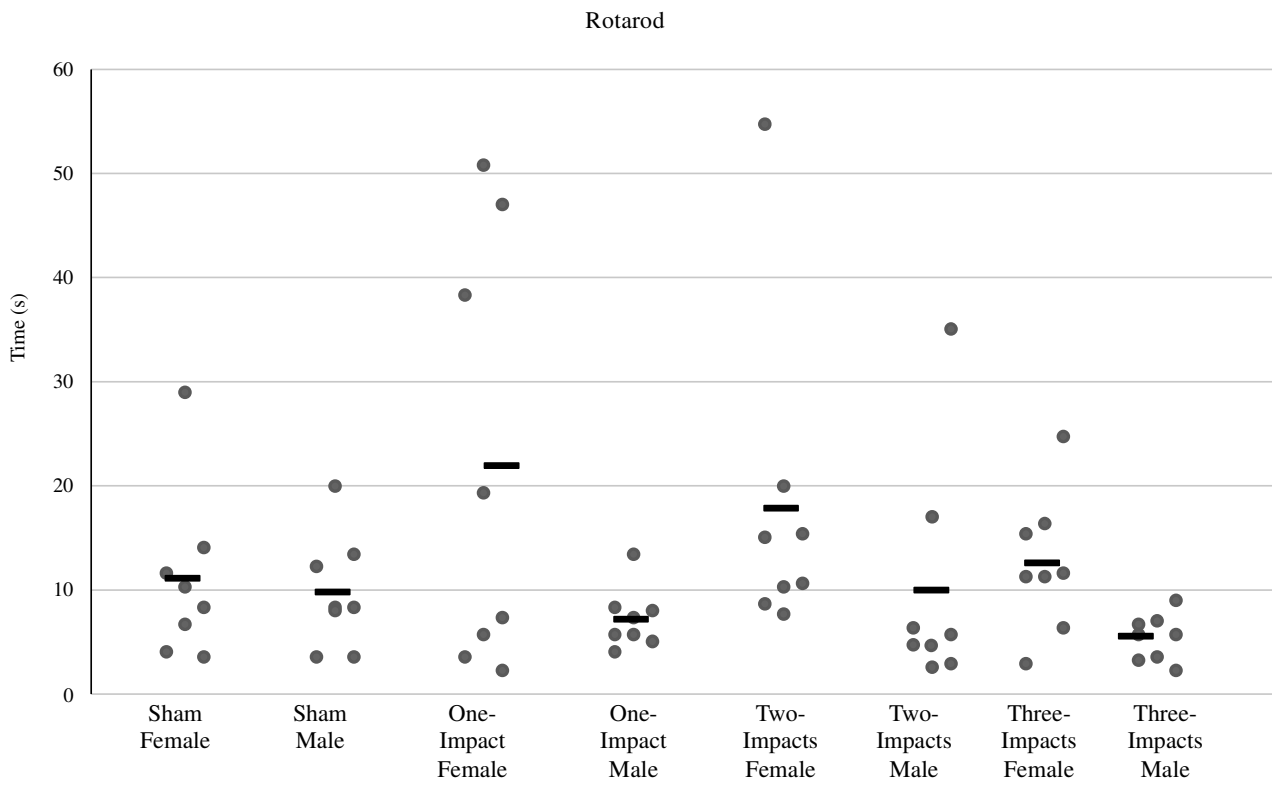

Figure 3. Time in seconds to remain on the rotating rod. Individual animal scores are the grey dots and group means are black lines. There was a significant effect of sex, but further probing found no significant comparisons.

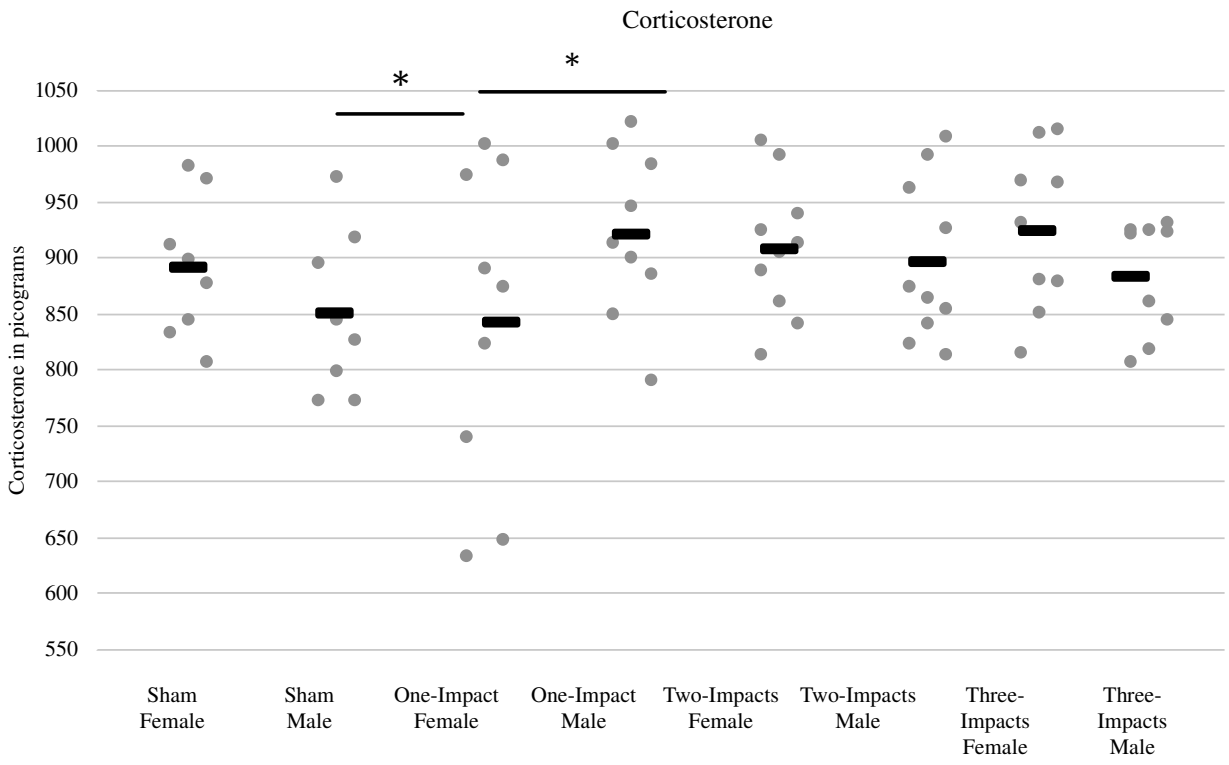

Figure 4. Corticosterone in picograms as collected from trunk blood. Individual animal protein levels are the grey dots and group means are black lines. The single-impact female group had the lowest level of corticosterone, compared to all other groups. Further probing revealed the single-impact females had significantly less corticosterone compared to the sham male and two-impact female groups (* indicates $p < 0.05$).

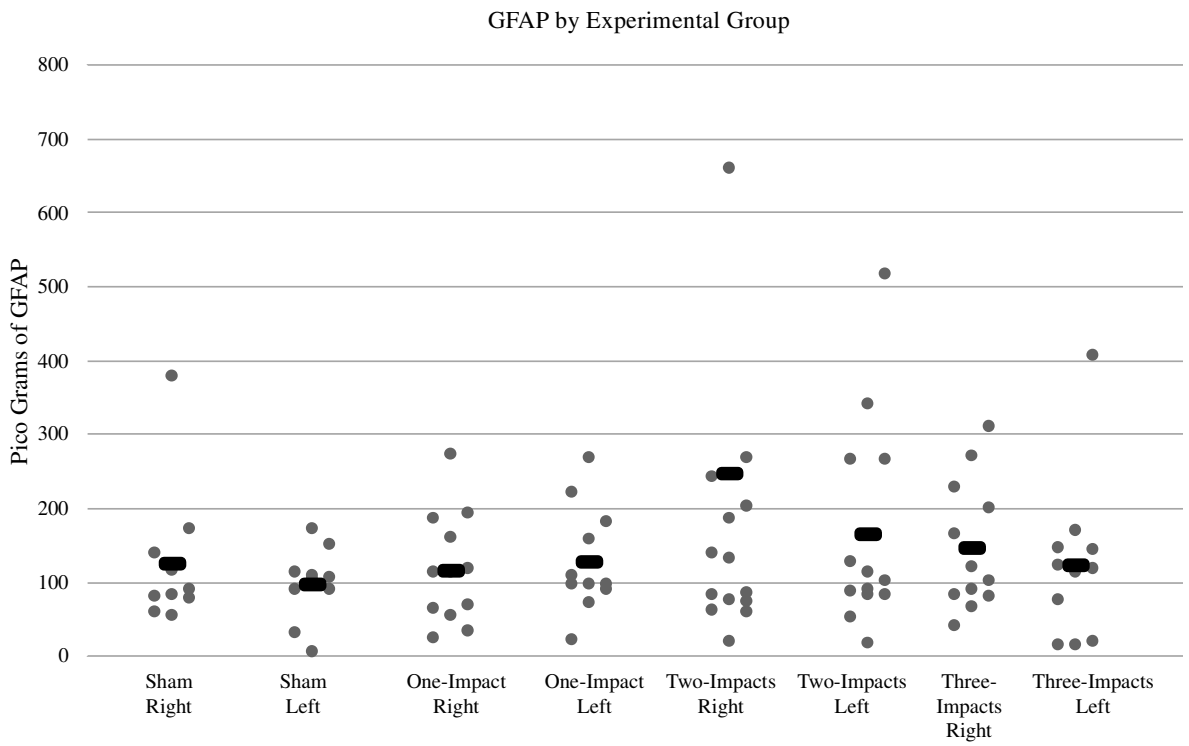

Figure 5. GFAP in picograms in left or right motor cortices. Individual animal protein levels are the grey dots and group means are black lines. We found no significant differences in GFAP levels. We ran the analyses with the possible outliers in the two-impact group removed, but did not results did not change therefore we kept all data.