A novel preclinical pediatric concussion model causes neurobehavioural impairment and diffuse neurodegeneration

By

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HBSc, Wilfrid Laurier University, 2013

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We acknowledge with respect the Lekwungen peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day

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Abstract

Concussions are the injury and symptoms that can result from transmission of a biomechanical force to the brain. They represent a significant global health burden, and are the subject of a growing body of medical research. A concussion can only be definitively diagnosed by a medical professional based on symptoms, although advanced neuroimaging and biomarker-based approaches are promising future diagnostic tools. There is no treatment for concussion beyond following return-to-work or -play guidelines, which recommend avoiding strenuous physical and cognitive activities until they no longer exacerbate symptoms. Preclinical models of concussion have been used to examine pathophysiological processes underlying symptoms, which is an important step in developing tools for diagnosis and treatment. Historically the clinical translation of preclinical concussion research has been limited, and the use of anaesthesia, and preference for adult male rats may contribute to this. These means of reducing variability are justified, but preclinical research moving forward should address these limitations to translatability by including more clinically relevant subjects and avoiding anaesthesia. To this end, we developed a new preclinical model for pediatric concussion. Our awake closed head injury (ACHI) model is well-suited to this purpose because it produces a helmeted closed-head injury involving vertical and rotational displacement of the head, and does not require anaesthesia. Before the ACHI model can be used to investigate concussion mechanism, diagnosis, and treatment, it needs to be characterized to demonstrate that it produces clinically relevant neurobehavioral and pathological changes. We developed a modified neurologic assessment protocol to test neurologic function immediately after each injury. The Barnes maze, elevated plus maze, open field, and Rotarod were used to measure injury-related changes in cognition, anxiety, and motor function. The Barnes maze reversal task was used to detect more subtle cognitive impairments of executive function. Structural MRI was used to search for visible lesion, hemorrhage, or atrophy; and silver-stain histology was used to detect neurodegeneration. We determined repeated ACHI produced acute neurologic impairment with the NAP, and a mild spatial learning deficit potentially mediated impaired cognitive flexibility in the Barnes maze and reversal training. These were accompanied by neurodegeneration in the optic tract, hippocampus, and ipsilateral cortex during the first week of recovery. Thus, following the internationally recognised definition developed by the concussion in sport group, we demonstrated 1) an “impulsive” force transmitted to the head results in 2) the rapid onset of short-lived neurologic impairment that resolves spontaneously. This occurs 3) with normal structural neuroimaging, and 4) produces cognitive impairment, and LOC in a subset of cases. The ACHI model is the first in Canada to forego anaesthesia, and this is the first demonstration of neurocognitive impairment accompanied by diffuse neurodegeneration in the absence of structural MRI abnormalities after mild traumatic brain injury in juvenile male and female rats.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

4xACHI	**four awake closed head injuries**
8xACHI	**eight awake closed head injuries**
Aβ	**amyloid beta**
ACHI	**awake closed head injury**
AD	**Alzheimer's disease**
ANOVA	**analysis of variance**
APP	**amyloid precursor protein**
βAPP	**beta amyloid precursor protein**
BACE1	**beta amyloid precursor protein cleaving enzyme 1 / beta secretase**
CC	**cage control**
CISG	**concussion in sport group**
CRT	**concussion recognition tool**
CT	**computerised tomography**
DTI	**diffusion tensor imaging**
DWI	**diffusion weighted imaging**
EPM	**elevated plus maze**
FA	**fractional anisotropy**
GFAP	**glial fibrillary acidic protein**
LOC	**loss of consciousness**
MD	**mean diffusivity**
MRI	**magnetic resonance imaging**
mTBI	**mild traumatic brain injury**
NAP	**neurologic assessment protocol**
NFT	**neurofibrillary tangles**
OF	**open field**
PID	**post-injury day**
PND	**postnatal day**
ROI	**region of interest**
SCAT5	**sport concussion assessment tool 5th edition**
S100β	**S100 calcium-binding protein beta**
TBI	**traumatic brain injury**
TWI	**track weighted imaging**
UCH-L1	**Ubiquitin C-terminal hydrolase-L1**

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Dedication

To the rats.

Chapter 1 -Introduction

1.1 Defining Concussion

Concussion is a term used to clinically describe the immediate and transient symptoms of a mild traumatic brain injury (mTBI) (McCrory, Feddermann-Demont, et al., 2017). Concussions can result from any biomechanical force transmitted to the brain (Ellis, Bauman, Cowle, Fuselli, & Tator, 2019; McCrory, Feddermann-Demont, et al., 2017). Acute neurologic abnormalities and short-lived loss of consciousness can occur at the time of injury (Castile, Collins, McIlvain, & Comstock, 2012; Charyk Stewart, Gilliland, & Fraser, n.d.; Guskiewicz, Weaver, Padua, & Garrett, 2000; Marshall, Guskiewicz, Shankar, McCrea, & Cantu, 2015). Concussions do not involve skull fracture or significant bleeding in the brain, which are signs of a more severe traumatic brain injury (McCrory, Meeuwisse, et al., 2017; Teasdale & Jennett, 1974). Instead, they are thought to involve microscopic damage and metabolic changes that manifest as a variety of symptoms in the following days to weeks. These symptoms can include headache, cognitive deficits, motor and reflex impairment, vision abnormalities, sleep disturbance, and numerous others (Ellis et al., 2019; Christopher C Giza & Hovda, 2014; McCrory, Feddermann-Demont, et al., 2017; Polinder et al., 2018; Theadom et al., 2016). Concussions are extremely heterogeneous injuries, with great individual differences in symptom severity and duration (Polinder et al., 2018). Notably, they occur in the absence of any visible focal lesion and cannot be detected with typical neuroimaging. Although they are categorized as *mild*, those who have been diagnosed with concussion often do not perceive their symptoms as mild.

Owing to the variability in etiology and outcomes, and the absence of visible injury, defining concussion has posed a historical challenge. The experiments described here are based on the definition for sport-related concussion (SRC) developed by the Concussion in Sport Group (CISG). These criteria were first established in 2001 by an international group of medical and research professionals with extensive concussion expertise (Aubry, 2002), and have been refined several times into their fifth iteration (CISG 5) (McCrory, Meeuwisse, et al., 2017). CISG 5 defines concussion as follows:

> Sport related concussion is a traumatic brain injury induced by biomechanical forces. Several common features that may be utilised in clinically defining the nature of a concussive head injury include:
>
> 1. SRC may be caused either by a direct blow to the head, face, neck or elsewhere on the body with an impulsive force transmitted to the head.
> 2. SRC typically results in the rapid onset of short-lived impairment of neurological function that resolves spontaneously. However, in some cases, signs and symptoms evolve over a number of minutes to hours.
> 3. SRC may result in neuropathological changes, but the acute clinical signs and symptoms largely reflect a functional disturbance rather than a structural injury and, as such, no abnormality is seen on standard structural neuroimaging studies.
> 4. SRC results in a range of clinical signs and symptoms that may or may not involve loss of consciousness. Resolution of the clinical and cognitive features

typically follows a sequential course. However, in some cases symptoms may be prolonged.

The clinical signs and symptoms cannot be explained by drug, alcohol, or medication use, other injuries (such as cervical injuries, peripheral vestibular dysfunction, etc.) or other comorbidities (e.g., psychological factors or coexisting medical conditions) (McCrory, Meeuwisse, et al., 2017).

This definition is endorsed by the Canadian Guideline on Concussion in Sport (Parachute, 2017), which was developed as an education guide for the recognition, diagnosis, and management of suspected concussions sustained in an athletic exposure. It is also endorsed by several international or national governing bodies for contact sports including American football, Australian football, basketball, cricket, equestrian sports, soccer, ice hockey, rugby league, rugby union, and skiing (Patricios et al., 2018). This demonstrates an international interest in developing uniform concussion diagnosis and management. A globally consistent definition facilitates collaborative research across borders. Although these documents define *sport* related concussion, authors emphasize that the diagnostic and management guidelines are broadly applicable to concussions that occur outside of sport as well.

It is important to distinguish a concussion, which is categorised as a mild traumatic brain injury (TBI) from a moderate or severe TBI. They can be distinguished with structural neuroimaging, where moderate or severe TBI may have a visible lesion, but mTBI does not. mTBI typically involves short-lived LOC of less than 30 minutes, if at all; altered mental state and post-traumatic amnesia for less than 24 hours; and a maximum Glasgow Coma Scale (GCS) of 13-15 within 24 hours of injury. In comparison, moderate TBI is characterized by 30 minutes

to 24 hours of loss of consciousness (LOC); greater than 24 hours of altered mental state; 1-7 days of post-traumatic amnesia; and a GCS maximum score of 9-12 in the first 24 hours of recovery. Severe TBI involves greater than 24 hours LOC; greater than 24 hours of an altered mental state; greater than 7 days of post-traumatic amnesia; and a maximum GCS below 9 within 24 hours of recovery (categorisation reviewed in (Blennow et al., 2016)).

1.1.1 Post-Concussion Syndrome

The majority of concussions resolve spontaneously, with a graded recovery lasting seven days to a month (Ellis et al., 2019; McCrory, Meeuwisse, et al., 2017). In a subset of cases referred to as post-concussion syndrome (PCS), symptoms can last for months or years (Hiploylee et al., 2017; McMahon et al., 2014; Polinder et al., 2018; Theadom et al., 2016; Voormolen et al., 2019). One study found that patients with symptoms persisting longer than three years never recovered (Hiploylee et al., 2017). Differing definitions of PCS provided by the Diagnostic and Statistical Manual, International Classification of Diseases, and Rivermead Post Concussion Symptoms Questionnaire make it difficult to estimate the incidence, because studies using differing diagnostic criteria find different incidences (Voormolen et al., 2018). A study that used these different criteria to diagnose PCS in a group with concussion history found the rate of PCS ranged from 6 to 38% depending on the criteria used (Voormolen et al., 2018). The reasons why some individuals are more vulnerable to long-term impairment remains under investigation, but white matter damage appears to contribute to PCS pathology (Khong, Odenwald, Hashim, & Cusimano, 2016; Messé et al., 2012). PCS can have significant social and professional consequences in addition to causing daily distress. Continuing research is needed to determine how to predict, prevent, and mitigate PCS.

1.2 Epidemiology and Etiology of Concussion

A World Health Organisation report estimates 600/100,000, or 420 million people sustain a concussion annually, and this accounts for between 70 and 90% of all traumatic brain injuries (Cassidy et al., 2004). The report found a global annual average of 300/100 000 individuals received treatment in a hospital for a concussion, but emphasize that this underestimates the true incidence because not all who sustain a concussion seek out medical attention. This is one of several challenges associated with estimating concussion incidence, along with regional differences in diagnostic criteria, diagnosticians, healthcare access, and niche populations available for study (e.g., athletes). Hon et al. suggest that these differences underlie the substantial regional disparities in concussion incidence reported in a global review of 11 concussion epidemiology studies (2019). They note that the majority of research comes from Canadian and American populations, and more studies outside of North America are needed to accurately estimate global concussion incidence.

The Canadian National Health Population Survey found 110/100000 Canadians reported a concussion as their most serious injury in the last 12 months (Gordon, Dooley, & Wood, 2006). An alarming Ontario study found an average of 147, 815 individuals, or 1153/100 000 of the population were diagnosed with concussion *annually* between 2008 and 2016 (Langer, Levy, & Bayley, 2020). A middle value of these estimates, for example 500/100 000 concussions per year, would represent a new concussion every three minutes in Canada. Concussion incidence appears to be increasing, although this may be due in part to increased public recognition resulting in more individuals seeking out medical attention (Langer et al., 2020). The rise in public recognition has accompanied increasing awareness that contact sports can put an athlete

at risk for long-term disability due to repeated concussions. Indeed, sports are the most common cause of concussion, and at least half of concussions occur during a sporting event or practice (Cassidy et al., 2004; Faul, Wald, Xu, & Coronado, 2010; Haarbauer-Krupa et al., 2018; Hon et al., 2019). Recent attempts to quantify head impact exposure in college athletes have indicated that football players experience an average of 6.3 head or body impacts per practice, and 14.3 impacts per game (Crisco et al., 2010). In the USA there are an estimated 1.6-3.8 million sports-related concussions per year (Daneshvar, Nowinski, McKee, & Cantu, 2011; Harmon et al., 2013; Langlois, Rutland-Brown, & Wald, 2006). A Canada-wide study of 5223 hockey players age 10-25 years old found 22% of athletes self-reported having sustained at least one concussion in their lifetime (Renton, Howitt, & Marshall, 2019). This rate is much higher than the 1.2% found in the non-sport-specific Ontario study (Langer et al., 2020), and the 6% WHO global estimate (Cassidy et al., 2004). These disparities suggest that athletes represent a higher-risk population for sustaining a concussion.

Increased recognition of SRC has led to public consideration of the ethical implications of professional athletes risking their health for entertainment. Clinical and pre-clinical concussion research is needed to develop diagnostic and treatment strategies to help athletes and other stakeholders make more informed decisions about their participation in contact sports in the context of their personal concussion risk. A positive outcome of this has been increased resources for concussion detection and management in professional sports. However, concussions also pose a great threat to non-professional and young athletes, who do not receive financial compensation or have access to the same high-quality educational, diagnostic, screening, and rehabilitation resources as professional athletes. For example, insufficient or

poorly fitted equipment may increase the likelihood of sustaining a concussion, whereas coaching on proper falling and player contact technique may reduce the likelihood of sustaining a concussion. Ongoing preclinical and clinical research should aim to develop efficient accessible diagnostic and treatment strategies that can be used in non-professional and non-athlete populations.

Athletic populations are common in concussion research, but epidemiology findings from exclusively athlete sample populations need to be taken in context. Contact sports teams provide convenient samples of individuals that can be expected to have a high number of exposures to impacts that could cause a concussion throughout the season, and they often share demographic traits relevant to controlling experiments. Findings from these studies have informed an increasingly detailed understanding of trends in SRC, but they may be less relevant to non-athlete populations.

An interesting confound that has arisen in clinical sport concussion research is athletes deliberately concealing their concussions. Return-to-play recovery guidelines mandate athletes with suspected concussion be removed from games and practices as long as participation exacerbates symptoms (Parachute, 2017). To avoid being removed from play, they may lie about self-reported symptoms, or perform purposely poorly during baseline analysis. This highlights the need for ongoing clinical and pre-clinical research to develop more objective diagnostic strategies and effective treatments.

Outside of contact sports, other common causes of concussion include combat exposure, vehicular and bicycle accidents, workplace accidents, assault including intimate partner violence, and falls (Cassidy et al., 2004; Faul et al., 2010; Haarbauer-Krupa et al., 2018; Hon et al.,

2019; McCrory et al., 2013). Importantly, many of these are events that an individual has limited capacity to take preventative action against. For example, it is not reasonable to expect never to experience an accident. From a public health and research perspective, this means preventive medicine cannot be the primary strategy to address concussions, and effective diagnosis and treatment must be a priority. Additional pre-clinical research is needed to develop such diagnostic and treatment technologies.

1.3 Risk Factors for more Severe Outcomes

Several risk factors have been identified for concussion. Ongoing research is needed to understand why these populations are at higher risk for sustaining a concussion, or for experiencing more severe and persistent symptoms. Identifying new risk factors may help to identify concussion in previously under-recognised populations, and to target research, education, and treatment resources where they can be most efficiently used.

1.3.1 Repeat injury

Concussion history is a risk factor for sustaining an incident concussion, also known as repeated mTBI (Barkhoudarian, Hovda, & Giza, 2011; HIDES et al., 2017; Tremblay et al., 2013; Tsushima, Siu, Ahn, Chang, & Murata, 2019; Van Pelt et al., 2019; Zemper, 2003). This may be due to lifestyle and environmental factors that predispose an individual to this type of injury, or to deficits caused by the initial injury (Guskiewicz et al., 2003; McCrory et al., 2013). Those who have had multiple concussions tend to experience more severe and persistent symptoms (Guskiewicz et al., 2003; Oyegbile, Dougherty, Tanveer, Zecavati, & Delasobera, 2020). Repeated mTBI is associated with learning and memory impairment (Bijur, Haslum, & Golding, 1996; Matser, Kessels, Jordan, Lezak, & Troost, 1998; Wall et al., 2006), slowed balance recovery

(Slobounov, Slobounov, Sebastianelli, Cao, & Newell, 2007), impaired visuospatial perception (Matser et al., 1998), difficulty in concentration, and increased incidence of headaches (Gaetz, Goodman, & Weinberg, 2000). While symptoms of a single concussion are more likely to resolve spontaneously, repeated injuries are more likely to cause symptoms to persist for extended periods (Arciniegas, Anderson, Topkoff, & McAllister, 2005; Halstead & Walter, 2010; Pellman, Viano, Tucker, Casson, & Waeckerle, 2003). Moreover, increasing evidence suggests a link between repeated mTBI and increased risk of developing dementia (Guskiewicz et al., 2005) and other neurodegenerative diseases (Masel & DeWitt, 2010; McKee et al., 2009). This is especially problematic for athletes in contact sports, military, or workers in other occupations with greater rates of exposure to physical injury. Given the lack of treatment options, they may be left with a difficult choice knowing that maintaining athletic and occupational commitments puts them at risk for multiple concussions. More research is needed to determine why cumulative damage by repeated concussions can produce worse outcomes.

1.3.2 Sex and Gender

Clinical studies agree there are sex difference in the incidence and severity of concussion, but findings are variable with resect to which sex experiences worse outcomes. Most studies find females are more likely to experience higher incidence (Black, Sergio, & Macpherson, 2017; Cnossen et al., 2018; T. Covassin, Elbin, Harris, Parker, & Kontos, 2012; Tracey Covassin, Savage, Bretzin, & Fox, 2018; Kraus & Nourjah, 1988; Scopaz & Hatzenbuehler, 2013; Styrke, Sojka, Björnstig, Bylund, & Stålnacke, 2013; Van Pelt et al., 2019), and more severe and persistent symptoms (Bretzin et al., 2018; Broshek et al., 2005; Tracey Covassin, Moran, & Elbin, 2016; Oyegbile, Delasobera, & Zecavati, 2017; Silverberg et al., 2015),

but others show males experience higher incidence (Cassidy et al., 2004; Rosene et al., 2017). Some studies find no difference in concussion prevalence between males and females(Renton et al., 2019). Two studies have found that across multiple sports males were more likely to sustain a concussion overall, but within sports that had equivalent governance and rules regarding contact in male and female leagues, females were more likely to sustain a concussion (Bretzin et al., 2018; Marar, McIlvain, Fields, & Comstock, 2012). A systematic review of sex-differences after concussion noted that outcomes were variable with respect to the type and severity of symptoms experienced (Merritt, Padgett, & Jak, 2019). A recent meta-analysis of 38 concussion incidence studies found incidence was higher in females in soccer and basketball, but there was no sex difference in incidence in baseball, hockey, lacrosse, swimming, or track (J. Cheng et al., 2019).

Sex differences in concussion outcomes reflect physical factors including physiology and biomechanics, as well as gendered differences in socio-cultural factors like symptom reporting, and method of injury (Daneshvar et al., 2011). Within similar sports, males are more likely to sustain concussions resulting from player to player contact, while female athletes are more likely to sustain a concussion resulting from contact with a playing surface or object (Chandran, Barron, Westerman, & DiPietro, 2017; Dick, 2009). The local social and cultural context should be taken into account when considering how societal gender roles explain sex differences in concussion risk factors on a clinically relevant individual basis. For example, gendered differences in contact sport participation or governance may be driven by regional cultural norms. Similarly, cultural expectations of stoicism in males may differentially discourage

discussion of discomfort or personal injury, especially in cases like concussion where the injury is not immediately visible. This can lead to under-reporting of concussion in males.

There are also physical sexual dimorphisms that contribute to sex differences in concussion outcome. On average, males tend to have greater muscle density and strength, including in the neck, and a smaller head to body ratio. This might make them more resilient to head movement and resultant damage during a rapid acceleration or deceleration (TIERNEY et al., 2005). While biomechanical differences appear to favour males, they may be masked by hormonal differences, as estrogen appears to be neuroprotective after TBI (Kövesdi, Szabó-Meleg, & Abrahám, 2021; Naderi, Khaksari, Abbasi, & Maghool, 2015). Notably, one study showed the majority of concussions occurred during the luteal phase of the menstrual cycle in female athletes (La Fountaine, Hill-Lombardi, Hohn, Leahy, & Testa, 2019). During this phase, estrogen levels are diminishing and reach the cyclical minimum. With respect to brain microstructure, sexual dimorphisms have been identified in neurogenesis, neuron morphology and distribution, and synaptic plasticity (reviewed in (Choleris, Galea, Sohrabji, & Frick, 2018; Sheppard, Choleris, & Galea, 2019), all of which may be differentially affected by concussion.

1.3.3 Age

Age can affect concussion risk and symptom severity. Concussion incidence is higher in children and adolescents than middle-age adults (Cassidy et al., 2004; Tsushima et al., 2019; A. L. Zhang, Sing, Rugg, Feeley, & Senter, 2016). Younger age groups are also more prone to persistent symptoms compared to middle-aged adults (Tracey Covassin, Elbin, Harris, Parker, & Kontos, 2012; Field, Collins, Lovell, & Maroon, 2003; McCrory et al., 2013; Nelson et al., 2016b; Scopaz & Hatzenbuehler, 2013). Although these age-based differences are common findings,

they are not universal. Other studies have found no differences in concussion outcomes between high school and collegiate athletes (Lee, Odom, Zuckerman, Solomon, & Sills, 2013; Nelson et al., 2016a). Findings of age-based differences in concussion outcomes are often variable based on what type of symptoms are assessed. One study found emotional symptoms were worse in adults, but memory symptoms were worse in children (Tracey Covassin, Elbin, Larson, & Kontos, 2012). Emerging evidence also suggests older adults are vulnerable to more severe and persistent concussion symptoms than middle-age adults (Gardner, Dams-O'Connor, Morrissey, & Manley, 2018).

Age-based differences in concussion incidence and outcomes may be explained by behavioural differences. For example, adult athletes are typically more experienced. They may be able to better anticipate head-impacts during game play, and therefore position themselves to avoid it, or absorb the motion in a way that minimises damage to the head and brain (Mihalik et al., 2010). A study comparing collegiate and high school football players found concussion in high school players were more likely to result from player contact, whereas in colligate athletes concussions were more likely to result from contact with the playing field or game apparatus (Lynall, Campbell, Wasserman, Dompier, & Kerr, 2017).

Age-based differences in concussion outcomes involve a combination of behavioural, biomechanical, and physiological differences between young and old populations (Ommaya, Goldsmith, & Thibault, 2002). Biomechanical studies of concussion have suggested that since children's brains are smaller they require a greater application of force to sustain concussive damage than an adult brain (Ommaya et al., 2002). Children tend to have a larger head to trunk ratio, and relatively weaker neck muscles. These physical factors mean children are less able to

prevent the transmission of force and motion to the brain during an impact or rapid acceleration (Proctor & Cantu, 2000)..

The developing brain is a unique physiological environment, and responds differently to trauma than the adult brain. Damage sustained during a concussive injury will not only affect current structure and physiological processes, but may also affect developmental processes. Brain development continues into early adulthood in humans (Sowell, Thompson, Tessner, & Toga, 2001). Until then the brain is in a constant state of dynamic changes in structural and functional connectivity (Watson, DeSesso, Hurtt, & Cappon, 2006). Axonal myelination occurs throughout development, plateauing during adulthood (Levitt, 2003). This is noteworthy in light of a preclinical study that found unmyelinated axons are more vulnerable to injury, with greater injury-induced impairment of electrophysiological function compared to myelinated fibers (Reeves, Phillips, & Povlishock, 2005). Regional distributions of cell populations, including the distribution and function of immune cells, also change throughout development. Disruption of developmental processes may exacerbate long-term impairments after pediatric concussion.

1.4 Diagnosis

Concussions are diagnosed by a physician according to their assessment of observed signs and self-reported symptoms, which is facilitated by standardised assessments like the GCS or the sport concussion assessment tool (SCAT5) (Echemendia, Meeuwisse, McCrory, Davis, Putukian, Leddy, Makdissi, Sullivan, Broglio, Raftery, Schneider, Kissick, McCrea, Dvorak, Sills, Aubry, Engebretsen, Lossemore, Fuller, Kutcher, Ellenbogen, Guskiewicz, Patricios, Herring, et al., 2017; McCrory et al., 2013; Teasdale & Jennett, 1974). Objective

diagnosis can be challenging, despite increasing their incidence and notoriety in medical research (Zemek et al., 2017), because there are no definitive diagnostic tools for concussions (Ellis et al., 2019; McCrory, Feddermann-Demont, et al., 2017). They cannot be detected with standard neuroimaging scans like computed tomography (CT) or structural MRI (Hughes et al., 2004; McCrory et al., 2013). Biofluid-based concussion biomarkers and advanced diagnostic imaging techniques are able to detect physiologic and microscopic structural changes resulting from concussion (D. K. Wright, Trezise, et al., 2016), but currently concussion can only be formally diagnosed with symptomatic assessment by a medial professional (Ellis et al., 2019; C. C. Giza et al., 2013; McCrory, Meeuwisse, et al., 2017). This demonstrates a clear need for ongoing concussion research.

1.4.1 Symptoms

Since there is usually no visible injury associated with concussion, diagnosis is based on presentation of a constellation of any of a large group of common symptoms. The concussion recognition tool handily summarizes the most common and recognisable concussion signs and symptoms, which are shown in **Table 1** ("Concussion recognition tool 5©," 2017a)**.** The symptoms listed there are not exhaustive, but they are useful in early recognition since they may arise soon after injury. Any unexplained behavioural or physical symptom including mild impairment to neurologic, cognitive, reflexive, or sensorimotor function, which appears a short time after a head impact, may be a sign of concussion.

A positive outcome of increasing concussion education and awareness is that athletes are more likely to recognise when they have sustained one, and know what steps to take during recovery (McCrea, Broglio, McAllister, Zhou, et al., 2020). However, as concussion awareness

Table 1.1: Concussion signs and symptoms. The information here is extracted from the Concussion in Sport Group's Concussion Recognition Tool 5 ("Concussion recognition tool 5©," 2017b; McCrory, Meeuwisse, et al., 2017). *Red flags for more severe injury requiring emergency medical attention are highlighted***.

Symptoms	Headache or head pressure*	Confusion
	Neck pain	Feeling:
	Dizziness	"Slowed down"
	Nausea	"In a fog"
	Vomiting	"Not right"
	Sound sensitivity	Sadness
	Light sensitivity	Nervousness
	Vestibular impairment	Anxiety
	Fatigue: low energy and drowsiness	Irritability
	Difficulty concentrating	Increased emotion
	Difficult remembering	
Visible Signs	Loss of consciousness	Seizure
	Reduced responsiveness	Grabbing/ reaching at head
	Laying motionless on ground	Clutching head
	Slow to get up	Dazed, vacant expression
	Unbalanced or uncoordinated	Confusion about recent events or current situation
	Falling	
Memory	Failure to answer any of the following may suggest concussion***	
	"What venue are we at today?"	
	"Which half is it now?"	
	"Who scored last in this game?"	
	"What team did you play last game?"	
	"Did your team win the last game?"	
Red Flags**	Neck pain	Deteriorating/ loss of consciousness
	Increasing confusion or irritability	Severe or increasing headache
	Seizure or convulsion	Unusual behaviour
	Weakness or tingling/burning in limbs	Double vision

**Headache caused by concussion is heterogeneous, but typically mild to moderate, global, pounding, throbbing, or dull. Severe, thunderclap, or progressively worsening headache may be a sign of intracranial hemorrhage requiring emergency medical attention*(Ellis et al., 2019).

***If any red flag is reported, immediate assessment by a medical professional is required. If no medical professional is available, consider transpiration by ambulance for emergency medical assessment.*

****In non-sport injuries use contextually relevant orienting questions*

increases, a concurrent problematic trend is athletes masking symptoms in order to avoid a concussion diagnosis that removes them from game-play (Garrick et al., 2005). There are complex contexts that can predicate such an attempt to mask symptoms and continue playing. Ultimately this behaviour puts an individual at higher risk for sustaining a second concussion during a vulnerable recovery period. An important consideration in this trend is that executive dysfunction is a symptom of concussion (Kunker, Peters, & Mohapatra, 2020), meaning decision making may be impaired during the recovery period. In other words, concussion may impair an athlete's ability to consider the consequences of masking symptoms. Their ability to weigh the consequences of a short term removal from play, against long term disability that may be caused if they do not take time to recover, is impaired. Thus, an important goal for concussion diagnostics and management is to develop objective biomarkers that cannot be easily masked.

1.4.2 Biomarkers

Biological and computerised tests

A biomarker-based strategy is a promising approach for objective concussion diagnosis. There is likely no single biomarker that will definitively diagnose concussion, but convergent evidence from multiple biomarkers for concussion will provide a more objective diagnosis than symptom assessment alone(Costello, Kaye, O'Brien, & Shultz, 2018). Good potential biomarkers include consistent detectible biological changes that result from concussion. A growing list of potential blood-based biomarkers for concussion have been identified, and ongoing studies attempt to determine a combination of blood-based biomarkers that can be

assayed to reliably diagnose concussion (reviewed in (O'Connell et al., 2018; Papa, Ramia, Edwards, Johnson, & Slobounov, 2015)). For example, the NCAA and Department of Defense CARE consortium tested blood samples from collegiate athletes for changes in glial fibrillary acidic protein (GFAP), Ubiquitin C-terminal hydrolase-L1, neurofilament light chain (UCH-L1), and tau protein, which are potential molecular biomarkers that are typically found to have increased concentration in the blood TBI. They found GFAP, UCH-L1, and tau were significantly elevated in athletes that had been diagnosed with concussion (McCrea, Broglio, McAllister, Gill, et al., 2020). Conversely, a systematic review summarizing 4352 publications examining s100 calcium-binding protein β (S100β), tau, neuron-specific enolase , and GFAP as putative blood-based biomarkers found that S100β was the only molecule that reliably predicted concussion (O'Connell et al., 2018). An important limitation of biomarker analysis is that individual baseline measurements are often needed to detect subtle changes resulting from concussion, since most of these biomarkers are endogenously expressed in lower levels in individuals that have not been diagnosed with concussion. It is more often the change in expression of a biomarker rather than its absolute absence or presence that signifies brain trauma (Asken et al., 2018). Shortened telomere length may also be a biological detectible sign of concussion. In preclinical studies, neuronal telomeres were shortened in response to concussion, which correlated with a shortening of telomere length in epidermal cells (Hehar & Mychasiuk, 2016; D. K. Wright, O'Brien, Mychasiuk, & Shultz, 2018). This correlation is important because epidermal cells can be collected non-invasively.

The ability to be measured with minimally invasive methods is an important goal in developing concussion biomarkers. To this end, computerised tests of vision and reflexive

changes are another promising approach to concussion evaluation. The use of standardised assessment tools like the SCAT5 or CRT are ubiquitous in concussion assessment, and non-invasive, but because they must be administered by a medical professional (i.e. a human) the results can be affected by misunderstanding, bias, or inconsistent interpretation (Echemendia, Meeuwisse, McCrory, Davis, Putukian, Leddy, Makdissi, Sullivan, Broglio, Raftery, Schneider, Kissick, McCrea, Dvorak, Sills, Aubry, Engebretsen, Lossemore, Fuller, Kutcher, Ellenbogen, Guskiewicz, Patricios, Herring, et al., 2017). Similarly, computerised surveys of self-reported symptoms can be prone to bias or manipulation (Broglio et al., 2018). Computerised and automated tests of vision, balance, and coordination may be less susceptible to these issues, and have demonstrated capacity to differentiate between concussed and non-concussed individuals (Lysenko-Martin, Hutton, Sparks, Snowden, & Christie, 2020; Maruta, Spielman, Rajashekar, & Ghajar, 2018; Massingale et al., 2018). Such tests are useful because they are non-invasive and can even be entertaining, which helps increase patient engagement. These types of computerised and automated tests are limited because they may require apparatus that not all those with suspected concussion have access to (Holden et al., 2020). These also typically require a baseline performance comparison in order to diagnosis concussion on an individual basis. With ongoing refinement, computerised and automated tests are becoming increasingly valuable tools for diagnosing concussion, especially when used in combination with other biomarkers.

1.4.2.1 Advanced Diagnostic Neuroimaging

Concussions cannot be detected in typical structural neuroimaging (McCrory, Meeuwisse, et al., 2017). Computerised tomography (CT) and structural magnetic resonance

imaging (MRI) are typically only used to rule out a more severe injury, which would be denoted by any visible lesion or bleeding. More recently developed advanced neuroimaging methods are able to detect changes associated with concussion, but these methods require further refining before they are sensitive enough to provide conclusive concussion diagnosis. Diffusion weighted imaging (DWI) is an advanced MRI method that uses specialized software and filters to interpret the MRI signal to provide a visual representation of the relative probability of diffusion of water molecules within brain tissue (Alexander et al., 2011). Water molecules undergoing random Brownian motion diffuse more freely in the axis parallel to organized structures (axial diffusion), since diffusion is more restricted by cellular membranes and organelles in the perpendicular axes (radial diffusion) (Alexander, Lee, Lazar, & Field, 2007). Both axial and radial diffusion restriction in can be increased by tissue damage, and this can be detected using DWI (Shenton et al., 2012).

Diffusion tensor imaging (DTI) is a common application of DWI. While DWI measures the relative ease of diffusion of water molecules, DTI is able to derive the degree and direction of diffusion of water molecules (K. lin Xiong, Zhu, & Zhang, 2014). This directional diffusion information can be interpreted to create *in vivo* maps of white matter tracts (Alexander et al., 2007). Fractional anisotropy (FA) is one DTI measure, which describes the fraction of diffusion that is anisotropic. Anisotropic diffusion is much greater axially than radially, whereas isotropic diffusion occurs equally in all three axis. Water diffusion near white matter tracts is more anisotropic in the direction parallel to the tract (i.e. higher fractional anisotropy), and water diffusion in grey matter tends to be less anisotropic (Alexander et al., 2007). FA is sensitive to a variety of white matter abnormalities including changes in structural integrity, fiber density,

axonal caliber, and degree of myelination (Alexander et al., 2007; Wilde et al., 2008). Microscopic damage to white matter tracts can increase diffusion restriction in the axial plane, which reduces fractional anisotropy. Indeed, FA is frequently reduced after clinical concussion (Bazarian et al., 2007; Niogi et al., 2008; Toledo et al., 2012; Wilde et al., 2008; Yuh et al., 2014).

Mean diffusivity (MD) is another DTI measure that may be altered by concussion (Cubon, Putukian, Boyer, & Dettwiler, 2011; Toledo et al., 2012). While FA measures the proportion of diffusion that is axial, in order to determine a direction of diffusion, MD calculates the average magnitude of axial and radial diffusion, in order to determine the rate of diffusion. It is less sensitive to white matter changes, and more sensitive to changes in edema or cell proliferation (Alexander et al., 2007). Track weighted imaging (TWI) is a recent advance in in DWI which may be more sensitive to white matter pathology (Calamante, Tournier, Smith, & Connelly, 2012). It allows properties of the tractograph including density, curvature, and path length, to be manipulated in order to more closely examine structural pathologies. TWI estimates the contents of each individual voxel based on the continuity of information through long-distance fiber tracks traversing them (Pannek et al., 2011). Note that the fiber *tracks* described here refer to digitally rendered streamlines, and not to neural *tracts* in this context. This provides super-resolution (sub-voxel) structural information, and can be interpreted to measure white matter integrity (Calamante et al., 2012; Pannek et al., 2011). Although DWI cannot yet provide a definitive concussion diagnosis, it provides convergent evidence of injury along with fluid-based biomarkers and symptomatic assessment. Ongoing work to develop these techniques will refine them towards more individual utility in concussion diagnosis.

1.5 Management

To date no pharmaceutical, biotechnology, or medical device has been approved to reduce symptom duration or severity in concussion management (Ellis et al., 2019; McCrory, Meeuwisse, et al., 2017). Because there are no effective treatments for this type of injury, and intense exertion tends to exacerbate symptoms, recovery guidelines such as the Parachute framework (Parachute, 2017) used in Canada recommend that concussion patients avoid demanding physical and cognitive activities, until those activities no longer exacerbate symptoms (Asken et al., 2016; Brown et al., 2014; Ellis et al., 2019; C. C. Giza et al., 2013). They suggest a graded return to work or play, advocating for an immediate 24-48 hour total rest period, followed by a gradual increases in daily activities that do not aggravate symptoms (Ellis et al., 2019). Notably, the activity exposure in itself is an important part of recovery. Moderate exercise of an intensity that does not exacerbate symptoms can improve recovery outcomes (Leddy, Haider, Ellis, & Willer, 2018).Furthermore, concussion patients that completely avoid exercise for an extended period of time tend to have prolonged symptoms (Silverberg & Iverson, 2013). In fact, in healthy individuals, several days to a week of total bed rest can cause headache, restlessness, vestibular and mood disturbance, and difficulty sleeping; which are all analogous to concussion symptoms (Fortney, Schneider, & Greenleaf, 2011). Thus, it would appear excessive rest could confound concussion symptoms during recovery. In other words, if conservative approaches to return-to-work/play involve extended periods of total rest, the treatment itself could exacerbate symptoms. Symptoms are currently the main diagnostic indicator of recovery progress. This highlights the importance of having objective diagnostic options to track recovery.

Although concussion symptoms typically resolve spontaneously within the first week to month after the injury (Tracey Covassin, Elbin, & Nakayama, 2010; Ellis et al., 2019; Holmes, Chen, Yahng, Fletcher, & Kawata, 2020; McCrory, Meeuwisse, et al., 2017; Nance, Polk-Williams, Collins, & Wiebe, 2009), distress caused by symptoms, and by limitations imposed by return to work or play guidelines, can cause severe disruption to daily life during recovery (McMahon et al., 2014; Voormolen et al., 2019). An important goal of concussion research is to identify treatment methods that can significantly shorten concussion recovery time, and reduce symptom severity.

1.6 Animal Models of Concussion

Animal models of TBI are an important tool to help understand the pathophysiology of concussions, and for developing diagnostic and treatment strategies (Shultz et al., 2017). Modelling concussions in animals is a unique challenge because concussions are clinically identified as a constellation of symptoms, and there is no distinct macro-structural injury or pathology to replicate like other common disease or trauma models. Instead concussion models use some form of mechanically-induced brain disruption to produce a constellation of behavioural changes that relate to clinical concussion symptoms, and then examine the resulting pathophysiological changes. Several animal models have been developed to study TBI, and they have been instrumental in understanding how the brain reacts to trauma (Anthony L Petraglia, Dashnaw, Turner, & Bailes, 2014; Shultz et al., 2020, 2017; Y. Xiong, Mahmood, & Chopp, 2013). Four common types are weight drop, fluid percussion, controlled cortical impact, and impact acceleration deceleration (See (Y. Xiong et al., 2013) for a detailed review of each methodology).

While these models have provided the basis for a growing understanding of the complex neurometabolic changes that accompany TBI (Christopher C Giza & Hovda, 2014), it is important to acknowledge that technical aspects of some of these models, such as the surgical disruption of the skull and the use of anaesthesia may limit how these models can be used to understand the unique pathophysiology that results from mild closed head injuries (Flower & Hellings, 2012; Statler, Alexander, Vagni, Dixon, et al., 2006; Statler, Alexander, Vagni, Holubkov, et al., 2006). Historically, preclinical concussion models focused disproportionately on adult male subjects. Clinical studies show significant sex differences in concussion outcomes, and that younger age groups are a higher risk population. Representative animal models are needed to understand how concussion uniquely affects these higher risk groups. The historical focus on adult subjects in preclinical research is problematic because the developing brain may be more susceptible to traumatic damage, and injury may impair developmental processes in addition to brain function. Preclinical concussion models should be representative of all age groups, as this may allow for age-based optimization of clinical concussion management. To address this, rodent concussion models have been adapted for younger age groups (Eyolfson et al., 2020; Mychasiuk et al., 2014; Pham et al., 2021; Prins, Hales, Reger, Giza, & Hovda, 2011; White, Pinar, Bostrom, Meconi, & Christie, 2017). Similarly, the inclusion of female subjects in preclinical studies has increased (for example: (Eyolfson et al., 2020; Mychasiuk et al., 2014; D. K. Wright, O'Brien, Shultz, & Mychasiuk, 2017)). Findings from these models should translate better to a broader range of clinical populations.

The constellation of symptoms that arise after concussion are often complex, transient, and subtle. Individuals report that experiencing deficits intermittently, or only during greater

physical and cognitive challenges. Some of these subtle deficits may be difficult to recapitulate in rodent models because they involve advanced executive processing that is unique to humans. As well, concussion is a biomechanically induced injury, and more often results from impacts that produce a great rotational acceleration in the head. In humans, the cervical flexure of the brainstem means the ventral aspect of the brain and spine are on perpendicular axes, which are parallel in rodents. This limits the extent to which such rotational forces can be accurately replicated between species. As well, the rodent brain is lissencephalic, thus any human concussion pathologies caused by biomechanical forces unique to gyrencephalic structural organisation will not be reproduced in rodents. Although the translatability of information from animal models to clinical practices is be limited by these factors, animal models remain essential because they allow experimenters to investigate these injuries using controlled manipulations that are not possible in clinical studies.

1.6.1 Anaesthesia in preclinical concussion models

Until recently, anaesthesia has been used ubiquitously in animal models of concussion for ethical purposes, and to restrain the subject for precise impact targeting (Ahlers et al., 2012; A. Petraglia et al., 2014; Anthony L. Petraglia et al., 2014). This may be a problem because anaesthesia has known neuroprotective properties (Flower & Hellings, 2012; Gray, Bickler, Fahlman, Zhan, & Schuyler, 2005; List, Ott, Bukowski, Lindenberg, & Flöel, 2015; Luh et al., 2011; Patel, Drummond, Cole, & Goskowicz, 1995; Rowe et al., 2013; Statler, Alexander, Vagni, Dixon, et al., 2006; Statler, Alexander, Vagni, Holubkov, et al., 2006). This potential confound may contribute to difficulty with clinical translation of therapeutic strategies from previous models. Isoflurane is a common anaesthetic in animal concussion modelling. It significantly

improved motor function and reduced hippocampal neuronal death after experimental mild brain injury (Statler, Alexander, Vagni, Dixon, et al., 2006). Isoflurane is thought to be neuroprotective primarily because it increases vasodilation and reduces excitotoxicity, which are important mechanisms contributing to acute brain damage. Isoflurane also increases cerebral blood flow, which may reduce post traumatic hypo-perfusion (Hendrich et al., 2001) It might reduce excitotoxic damage by reducing glutamate release (Patel et al., 1995). Isoflurane also appears to inhibit N-methyl-D-aspartate receptors, which reduces intracellular calcium (Gray et al., 2005). These processes are implicated in the neurometabolic pathologies associated with concussion (Christopher C Giza & Hovda, 2014) In fact, anaesthetics are recommended in the clinical treatment of more severe head trauma to reduce overall damage and long term deficits by modulating intracranial pressure and cerebral metabolism (Flower & Hellings, 2012). Using anesthetics in experimental concussion may limit the clinical translatability of preclinical findings.

1.7 Concussion Mechanisms

There is no single mechanism on or pathological hallmark for concussion. Rather, these injuries reflect complex and heterogeneous involvement of multiple interconnected pathologies. These are often categorised as being part of either the primary or secondary injury. The primary injury occurs at the time of impact, and includes a rapid but short-lived physical, ionic, and metabolic disturbance brought on by mechanical tissue disruption. The secondary injury describes the cascade of pathophysiological processes that result from this initial disruption in the following minutes to weeks.

1.7.1 Primary Injury

The moment of impact produces an instant of dysregulation in the otherwise highly organized and tightly regulated brain. As brain tissue is rapidly displaced, neuronal soma, organelles, and processes, as well as glia and blood vessels are deformed. Tissue is compressed where the brain meets the skull. Structures are displaced differently depending on their size, shape, density, and connectivity. A cadaver study using high-speed biplane x-ray to measure brain displacement and deformation during concussive movement found the brain was displaced a maximum of 7mm relative to the skull (Hardy et al., 2007). This contributes to diffuse axonal injury, which is a common form of damage associated with concussion in which axons are twisted, torn, and sheared as a result of the rapid deformation of brain tissue (Romeu-Mejia, Giza, & Goldman, 2019). Complex microstructural elements such as dendrites, axons, and astrocytic processes are at higher risk for sustaining damage, as tension is applied to these fine processes when bulky soma are pulled or twisted away from distant terminals (Christopher C Giza & Hovda, 2014).

Cellular membranes and axolemma, only two molecules thick, may develop multiple sublethal defects through mechanoporation, which is the mechanical induction of microscopic holes in the membrane that may permit dysregulated ion flux (Christopher C Giza & Hovda, 2014). While axons are normally ductile and compliant during body movement, the rapid application of force that occurs during a concussion can cause the strained tissue to momentarily become brittle (Johnson, Stewart, & Smith, 2013; D. H. Smith, Wolf, Lusardi, M-Y Lee, & Meaney, 1999). This facilitates membrane damage in the form of microscopic holes that permits efflux of potassium ions, and influx of calcium and sodium ions (Romeu-Mejia et al.,

2019). Further, mechanosensitive sodium ion channels in neurons can be opened by the rapid tissue displacement, as membrane movement displaces anchored protein subunits associated with the channels, causing a structural change that allows influx of sodium ions through the channel (Maxwell & Graham, 1997; Wang et al., 2009). Increased intracellular sodium ion concentration can activate local voltage sensitive ion channels, and reverse the transport direction of sodium calcium ion exchange across the membrane, which increases intracellular calcium.

These multiple sources of calcium ion influx initiate depolarisation and dysregulated neuronal signalling, including excess glutamate release (Katayama, Becker, Tamura, & Hovda, 1990; Weber, 2012). ATP-driven membrane-bound pumps work in excess to restore membrane potential, resulting in excess ADP, hyperglycolysis, and a rapid depletion of energy reserves (Christopher C Giza & Hovda, 2014; Yoshino, Hovda, Kawamata, Katayama, & Becker, 1991). An acute state of hyperglycolysis is common after TBI, and may occur in neurons in response to increased activity (Manlio Díaz-García et al., 2017), or in astrocytes in response to increased glutamate uptake demands (Pellerin & Magistretti, 1994). Simultaneously, mitochondria sequester excess calcium as a method of restoring balance, which ultimately leads to widespread mitochondrial dysfunction (Weber, 2012). This exacerbates the metabolic and energetic crisis and impairs neuronal firing (Kim, Han, Gallan, & Hayes, 2017; Pivovarova & Andrews, 2010).

1.7.2 Secondary Injury

The initial hyperglycolytic state is relatively short lived (several hours) and is followed by a week to ten days of impaired glucose metabolism (Christopher C Giza & Hovda, 2014).

Studies have identified this hypometabolic state as a higher risk period for sustaining more serious injuries if a second impact is sustained during this time. This is accompanied by a period of global cerebral hypoperfusion, which may further exacerbate metabolic and energetic crisis by limiting metabolite transport and waste removal (Choe, 2016; Christopher C Giza & Hovda, 2014).

Neurodegeneration

Axonal Degeneration

Intra-axonal calcium flux can result in phosphorylation and resultant collapse of neurofilament side-arms, as well as proteolytic damage to other cytoskeletal components including spectrin (Pettus & Povlishock, 1996). Increased intracellular calcium activates calpain-mediated protease activity on microtubule associated proteins, which contributes to further microtubule destabilisation and a loss of axonal structural integrity (Weber, 2012). Physical disruption of microtubules interferes with bidirectional axonal transport of metabolites and neurotransmitters to and from the synapse, and can cause axonal disconnection in severe cases (Romeu-Mejia et al., 2019; Tang-Schomer, Johnson, Baas, Stewart, & Smith, 2012).

Another important outcome is detectable axonal accumulation of beta amyloid precursor protein (βAPP), which is a histological indicator of diffuse axonal injury (Gentleman, Nash, Sweeting, Graham, & Roberts, 1993; Johnson et al., 2013)**Cell Death**

The extent of apoptosis or necrosis that occurs after a single concussion is unclear. Preclinical studies of more severe or open-skull TBI frequently observe cell death using histological markers (Gao & Chen, 2011; Pullela et al., 2006), but this occurs inconsistently in mild injury models (Dikranian et al., 2008; Prins et al., 2011; Sauerbeck et al., 2018). Clinical

evidence has identified diffuse brain atrophy following repeat concussion (McCrea, Broglio, McAllister, Zhou, et al., 2020). In the most extreme cases such as professional athletes who sustain hundreds to thousands of head impacts throughout a lifetime, repeated head injuries can result in a progressive neurodegenerative disorder known as chronic traumatic encephalopathy (CTE) (Martland, 1928; Mez et al., 2017).

Neuroinflammation

One of the pathophysiological mechanisms thought to underlie concussion is maladaptive neuroinflammation (Corps, Roth, & McGavern, 2015). The inflammatory response activates specialized immune cells in response to trauma or infection. This promotes healing and reduces damage in most circumstances, but in the case of concussion neuroinflammation appears to contribute to pathology (Loane & Byrnes, 2010). Perturbation of ongoing neuroinflammatory processes is thought to contribute to the increased severity in symptoms experienced if an individual sustains a new concussion while recovering from a recent one (Aungst, Kabadi, Thompson, Stoica, & Faden, 2014).

The central nervous system is a unique immune environment because the blood brain barrier limits the infiltration of circulating immune cells (Ransohoff & Brown, 2012). Astrocytes and microglia are the primary resident immune cells of the CNS, and they mediate neuroinflammation after concussion (Hugh Perry & Teeling, n.d.; Myer, Gurkoff, Lee, Hovda, & Sofroniew, 2006; Ransohoff & Brown, 2012; J. a. Smith, Das, Ray, & Banik, 2012). Animal models of traumatic brain injury commonly report activation of microglia and astrocytes after injury (Aungst et al., 2014; Dhananjay R Namjoshi et al., 2014; A. Petraglia et al., 2014; Potts et al., 2006; Webster et al., 2015).

Microglia are CNS-specific macrophages (Hugh Perry & Teeling, n.d.). These motile cells can take on several distinct morphologies depending on their activation state, and have been extensively characterized (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). Ramified (resting) microglia move throughout brain tissue surveilling for signs of trauma or infection (Nimmerjahn, Kirchhoff, & Helmchen, 2005). They have a relatively small cell body and many long branching processes that constantly extend and retract as they sample their environment. If a sign of trauma or infection is detected they become activated, and they retract their processes and take on a more amoeboid shape. Activated microglia have phagocytic capabilities that allow them to remove dead and damaged cells and debris. Activated microglia increase expression of Iba1, which is a soluble cytosolic protein with actin-binding properties that modulates actin cytoskeletal elements to facilitate phagocytosis and cell migration (Ohsawa, Imai, Sasaki, & Kohsaka, 2004). They also release pro-inflammatory cytokines like interleukin 1, interleukin 6, and tumor necrosis factor α, which can recruit other microglia to the site of trauma (J. a. Smith et al., 2012), and stimulate activation of astrocytes (Klein, Möller, Jones, & Bluethmann, 1997).

Astrocytes have a complex role in the brain including providing metabolic and structural support to neurons (Y. Chen & Swanson, 2003), and regulating transportation of water, ions, and metabolites across the blood brain barrier (Sofroniew & Vinters, 2010). When they become activated they increase expression of GFAP – an intermediate filament protein that contributes to cyto-architectural dynamics (Pekny & Pekna, 2004). GFAP is commonly used as a marker to identify astrocytes, and changes in its expression are measured to detect their activation (Pekny & Nilsson, 2005; Pekny & Pekna, 2004). Activated astrocytes affect structural

plasticity after trauma chemically through the release of inflammatory cytokines, growth factors, and physically through the formation of glial scars that prevent neural regrowth (Corps et al., 2015; J. a. Smith et al., 2012; Sofroniew, 2009). Activated astrocytes are also thought to contribute to excitotoxicity through perturbations in their glutamate reuptake function (Obrenovitch & Urenjak, 1997; Rothstein et al., 1996). Reduced glutamate reuptake produces an excess of glutamate at the synapse that leads to over activation of glutamate receptors and deregulated neural firing (Christopher C Giza & Hovda, 2014). This can cause a neural metabolic crisis that culminates in cell death (Yi & Hazell, 2006). Excitotoxicity is a well-established mechanism of cell death in severe traumatic brain injury, but the extent to which it is present after mild injuries requires further investigation.

1.8 Summary of Project Aims

Preclinical research using animal models of concussion has provided an increasingly detailed understanding of the complex pathophysiological mechanisms that underlie concussions, but the clinical translation of putative diagnostic and treatment strategies identified in preclinical studies has been limited. This demonstrates a need for continued development of preclinical models to ensure they replicate the numerous etiology and symptomology associated with clinical concussion. To meet this need, we developed a novel preclinical model of pediatric concussion. Our awake closed head injury (ACHI) model is unique in that it does not require anaesthesia, and was designed for use in a juvenile population. Before the ACHI model can be used to investigate concussion pathophysiology, it must be characterized to demonstrate that it induces behavioural and structural changes analogous to clinical concussion symptoms.

We considered the CIGS5 definition of concussion, and aimed to show that the ACHI model 1) provides an "impulsive" force transmitted to the head, which 2) results in the rapid onset of short-lived neurologic impairment that resolves spontaneously. This 3) does not result in structural imaging abnormalities, and 4) results in evolving cognitive and clinical symptoms, and LOC in a subset of cases. Chapter 3 summarises our characterisation of acute neurologic, cognitive, and motor changes; anxiety; and structural MRI outcomes after single and repeated ACHI. In Chapter 4 we refined our characterisation of acute neurologic impairment, examined cognitive flexibility as a more subtle form of cognitive change, and performed histology to detect neurodegeneration after repeated ACHI.

Chapter 2 - General Methods

Methods in this chapter are published as follows:

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* I am listed as contributing equally with the other graduate students in the laboratory to reflect my exclusive role in developing the ACHI procedure in our lab; my development of the original NAP procedure upon which this refined scoring method was based; my collection of a portion of the NAP score data; and manuscript preparation.

Authors	Design	Concussion	Data Collection	Data Analysis	Manuscript Preparation
J.T.P	Yes	Yes	Yes	Yes	Yes
C.P.	Yes	Yes	Yes	Yes	Yes
K.J.N	Yes	Yes	Yes	Yes	Yes
A.M.	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
H.R.			Yes		
C.H.				Yes	Yes
B.R.C	Yes				Yes

Meconi, A.**, Wortman, R. C.**, Wright, D. K., Neale, K. J., Clarkson, M., Shultz, S. R., & Christie, B. R. (2018). Repeated mild traumatic brain injury can cause acute neurologic impairment without overt structural damage in juvenile rats. PloS One, 13(5), e0197187. https://doi.org/10.1371/journal.pone.0197187

**I am listed as co-first author with Ryan. R. Wortman to reflect his extensive contributions to the characterisation of this model in partial fulfillment of the requirement of his Masters thesis. A.M. was exclusively responsible for ACHI procedure development and primarily responsible for ACHI induction and NAP score collection, with assistance from R.C.W. A.M. was exclusively responsible for Barnes experiment. R.W. and K.J.N. optimized the open field, elevated plus maze, and Rotarod methods in our laboratory; A.M. collected and analysed these behavioural data. D.K.W and S.R.S. contributed all MRI scanning and analysis. M.C. contributed cresyl violet histology.

Authors	Design	Concussion	Data Collection	Data Analysis	Manuscript Preparation
A.M.	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
R.C.W.	Yes	Yes	Yes		Yes
D.K.W.	Yes		Yes	Yes	Yes
K.J.N.	Yes				Yes
M.C.	Yes		Yes		Yes
S.R.S.	Yes				Yes
B.R.C	Yes				Yes

2.1 ACHI procedure

We developed the ACHI model to produce a mild closed head injury in juvenile rats without the use of anaesthesia. It is adapted from procedures reported in adult mice (A. Petraglia et al., 2014; Anthony L. Petraglia et al., 2014). As shown in **Figure 2.1,** beginning on PND 25-28**,** rats were immobilized using clear plastic restraint cones (Model DC-200, Braintree Scientific, Braintree, MA). The cones have an opening at the nostril to provide ventilation, and are held closed behind the haunches using a plastic clip **(Fig. 2.1A, B)**. Custom 3D printed (Replicator-2, MakerBot, Brooklyn, NY; 1.75 mm ABS plastic filament) plastic helmets were used to help dissipate the force of the blow across the skull and reduce the chance of skull fracture **(Open arrows, Fig. 2.1A, B)**. The helmets were held in place with an elastic band and double-sided tape. The back of the helmet was aligned with the interaural line, and a flat circular surface (7 mm diameter) on the top of the helmet aided in targeting the impact over the left parietal cortex. A modified controlled cortical impact device (Impact One, Leica Biosystems Inc., ON, Canada) was mounted on a stereotaxic frame. The impactor was modified with the addition of a 7 mm diameter flat rubber tip (**Closed arrows, Fig. 1A, 1B**). The rats were placed on a soft foam platform (3" thick Super-Cushioning Polyurethane Foam Sheet, McMaster-Carr, OH) directly below the impactor. The impactor tip was carefully targeted over the left parietal cortex, and an electromagnetic piston drove the impact tip into the helmet at a speed of 6 m/s, and depth of 10 mm. The impactor was retracted immediately (100 ms dwell time) to prevent ricochet. After each impact rats were immediately removed from the restraint bag for assessment.

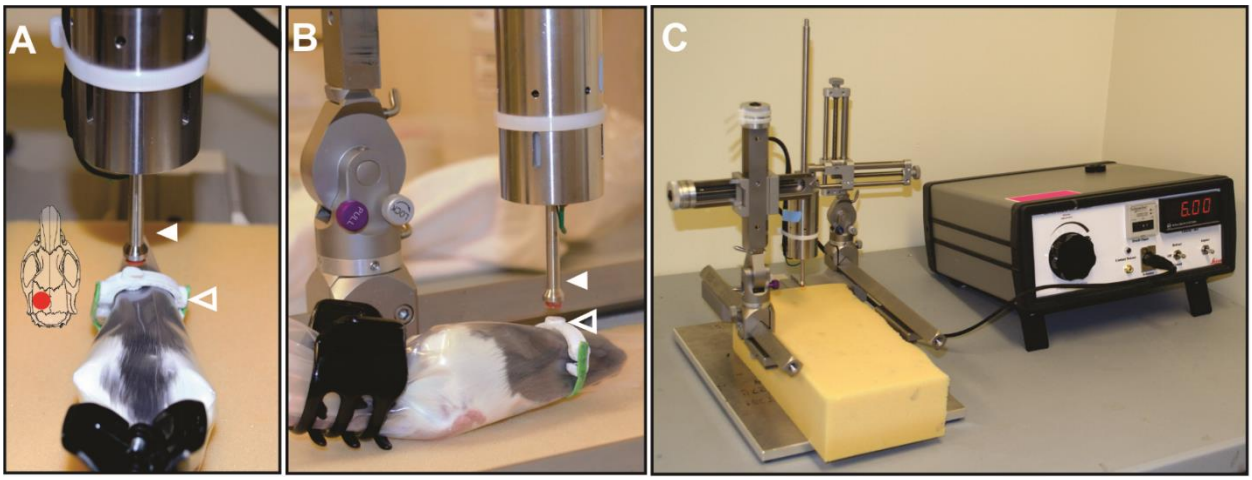


Figure 2.1 ACHI procedure and apparatus. (A,B) Rats were placed in a soft plastic restraint bag on a foam platform. A custom 3D printed helmet (open arrowhead) was placed on the head, with the impact site centered over the left parietal cortex. **(C)** A modified Leica Impact One controlled cortical impactor was used to generate the injury. It was modified by adding a 7 mm diameter rubber impact tip (closed arrowhead), and was set to a velocity of 6 m/s and dwell time of 100ms. The impactor was centered over the helmet target and adjusted to depress a depth of 10 mm, and the control box was used to initiate the impact.

2.2 Loss of Consciousness

Three common tests were performed immediately after each procedure to provide a convergent assessment of the animals' level of consciousness.

Apnea: After being removed from the restraint bag and placed upright on a clean surface each rat was initially examined for apnea. If they were not breathing, the amount of time from the start of the test until breathing returned was recorded as the latency to recovery.

Toe Pinch Reflex: The toe pinch reflex was then assessed by gently extending the rat's contralateral (to injury hemisphere) hind limb and pinching sharply and firmly. If the rat did not immediately retract the limb, the pinch was repeated at five second intervals on alternating hind limbs. The time from the first pinch until the rat retracted their limb was recorded as latency to recovery.

Righting Reflex: The righting reflex was determined by placing the rat on their back. The rat should immediately flip themselves upright, and if they did not, the amount of time taken for the rat to right themselves was recorded.

2.3 Neurological Assessment Protocol

The ACHI model uniquely allowed us to immediately perform a neurological assessment after each impact, without being delayed or affected by recovery from anesthesia. Similar to others, (Ding et al., 2013; Schaar, Brenneman, & Savitz, 2010; Shapira et al., 1988; Shohami, Novikov, & Bass, 1995) our NAP score assessed four basic neurological outcomes after each procedure. It consists of four simple reflexive and motor tasks, which can be tested within the first minute after the ACHI or sham. A different scoring method was used for each chapter, due to evolving improvements to this novel approach. In the Chapter 3 cohort a binary scoring method was used. Each task was scored as a pass or fail. **Table 2.1** shows representatives photographs of a control and impaired response in each task, as well as the scoring method used in the Chapter 4 cohort. The task criteria are described as follows:

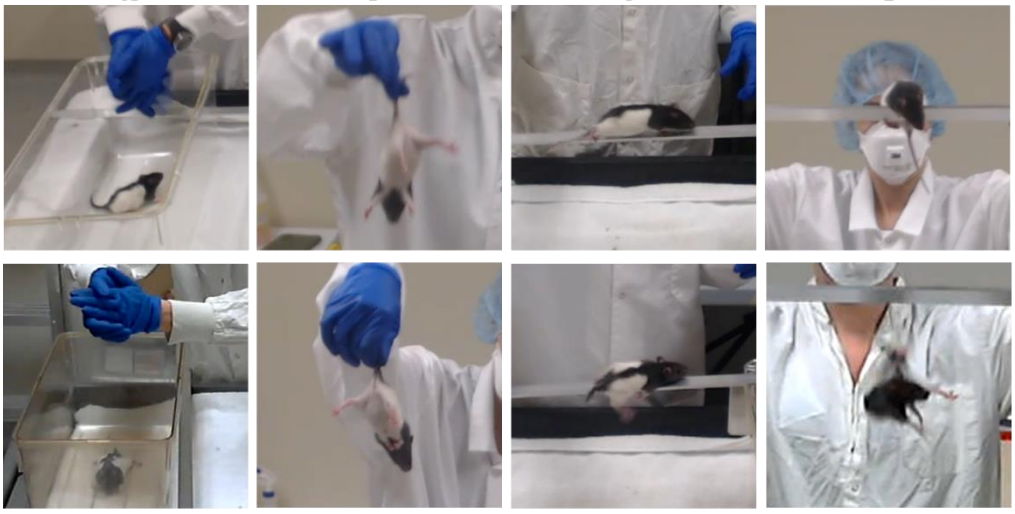
Startle Reflex: The rat is placed in the center of a clean, empty, standard housing cage, and the researcher claps loudly above the center of the cage. The rat should flinch, freeze, ear or tail twitch, blink, etc. in reaction to the clap. (**Table 2.1, A**)

Limb Extension: The rat is grasped by the base of the tail and raised 30-50 cm in the air to examine the limb extension response. The rat should fully extend both forelimbs, and a deficit is indicated if one or both are contracted or immobile (**Table 2.1, B**).

Balance Beam: The animal is placed on a flat narrow balance beam and their ability to balance and walk is assessed. The beam itself is 100 cm long x 2 cm wide x 0.75 cm thick, and is placed 22 cm above a cushioned work surface that extends from an empty cage to the animal's home cage. The rat is placed squarely balanced on the center of the beam, facing the home cage. Deficits are indicated by immobility, slipping or failing to grasp the beam with any limb, or falling from the beam. (**Table 2.1, C).**

Rotating Beam: The rat's ability to navigate a slowly rotating beam is assessed. The rat is placed squarely balanced on the center of the beam used in the previous task at a height of 75 cm above a cushioned work surface, and the beam is rotated once per second for 4 rotations. Falling from the beam indicates impairment **(Table 2.1, D).**

Table 2.1: Scoring criteria for Neurologic Assessment Protocol



Startle (reaction to clap)	Limb Extension	Beam Walk	Rotating Beam (successful rotations)	Score	Neurologic Impairment
No reaction	Limp Absence of postural tone	Immobility Hanging limbs	<1	**0**	Severe
Ear twitch	Intermittent retraction or clenching of limbs	Nonlocomotive movement "Swimming"or "Rowng"	1-2.9	**1**	Moderate
Slow reaction Slight Freeze	One limb extends, other is impaired	Locomotive movement with greater than 2 foot slips	3-3.9	**2**	Mild
Jump Freeze	Full extension of both limbs Actively grasp object	Locomotion to home cage with 2 or fewer foot slips	4	**3**	No impairment

Chapter 3 - Repeated ACHI caused acute neurocognitive impairment without structural MRI abnormalities

Experiments in this chapter are published as follows:

Meconi, A.**, Wortman, R. C.**, Wright, D. K., Neale, K. J., Clarkson, M., Shultz, S. R., & Christie, B. R. (2018). Repeated mild traumatic brain injury can cause acute neurologic impairment without overt structural damage in juvenile rats. PloS One, 13(5), e0197187. https://doi.org/10.1371/journal.pone.0197187

**I am listed as co-first author with Ryan. C. Wortman to reflect his extensive contributions to the characterisation of this model in partial fulfillment of the requirement of his Masters thesis. AM. was responsible for ACHI development. AM. was primarily responsible for ACHI induction and NAP score collection, with assistance from RCW. AM. was exclusively responsible for Barnes experiment. RW. and KJN. optimized the open field, elevated plus maze, and Rotarod methods in our laboratory; AM. collected and analysed all behavioural data. DKW and SRS. did all MRI scanning and analysis. MC. contributed cresyl violet histology.

Authors	Design	Concussion	Data Collection	Data Analysis	Manuscript Preparation
A.M.	Yes	Yes	Yes	Yes	Yes
R.C.W.	Yes	Yes	Yes	Yes	Yes
D.K.W.	Yes		Yes	Yes	Yes
K.J.N.	Yes				Yes
M.C.	Yes		Yes		Yes
S.R.S.	Yes				Yes
B.R.C	Yes				Yes

3.1 Chapter Abstract

Repeated concussion is becoming increasingly recognized as a serious public health concern around the world. As is the potential for repeated pediatric concussions to detrimentally alter the structure and function of the developing brain. To better study this issue, we developed an awake closed head injury (ACHI) model that enabled repeated concussions to be performed reliably and reproducibly in juvenile rats. A neurological assessment protocol (NAP) score was generated immediately after each ACHI to help quantify the effects of repeated injury on level of consciousness, and basic motor and reflexive function. We show that repeated ACHI (4 impacts in two days) can be administered in male and female juvenile rats without significant mortality or distress. We found that both single and repeated injuries produced acute neurological deficits resembling clinical concussion symptoms, which can be measured using the NAP score. Behavioural analyses found repeated ACHI acutely impaired spatial memory in the Barnes maze, and memory impairment correlated moderately with poorer NAP score performance in a subset of females. These cognitive impairments occurred in the absence of motor impairments on the Rotarod, or anxiety changes in the open field and elevated plus mazes. Magnetic resonance imaging (MRI) indicated that repeated ACHI did not produce visible structural damage or hemorrhage. MRI also confirmed there was no volumetric loss in the cortex, hippocampus, or corpus callosum of animals at 1 or 7 days post-ACHI. Together these data indicate that the ACHI model is a reliable, high throughput means to study the effects of concussions in juvenile rats.

3.2 Materials and Methods

3.2.1 Rats

All procedures used in this study were approved by the University of Victoria Animal Care Committee and are in compliance with Canadian Council for Animal Care guidelines. Juvenile Long Evans rats (n=94) were obtained from (Charles River Laboratories, St. Constant, PQ) or bred at the University of Victoria. Offspring were weaned at postnatal day (PND) 21 and housed in same-sex groups of 2-3. They were then assigned to one of three experimental groups (sham control, single ACHI, or repeated ACHI) so that no more than two rats from any one litter were assigned to any one experimental group. Average weight at the time of the first procedure was 70.0 g for males and 66.1 g for females. All rats were housed under standard laboratory conditions including automatically controlled temperature, humidity, ventilation and a 12-hour light/dark cycle with *ad libitum* food and water access. All purchased animals were allowed to adapt to the vivarium for at least one week prior to experimental procedures. After injury or behavioural testing animals were returned to their home cages unless otherwise specified. Two cohorts of rats were used for **Chapter 3**. Animals in cohort 1 were used for NAP testing, MRI analysis. Animals in cohort 2 were tested with the NAP, Barnes maze, Rotarod, elevated plus maze, and open field maze. Both cohorts followed the same injury timeline.

3.2.2 Experimental Timeline

The injury and experimental timeline used in **Chapter 3** are summarized in **Figure 3.1.** The ACHI and NAP procedure are described in detail in **Chapter 2.** Animals in the repeat ACHI group received two impacts per day, with a two-hour interval between injuries, for two

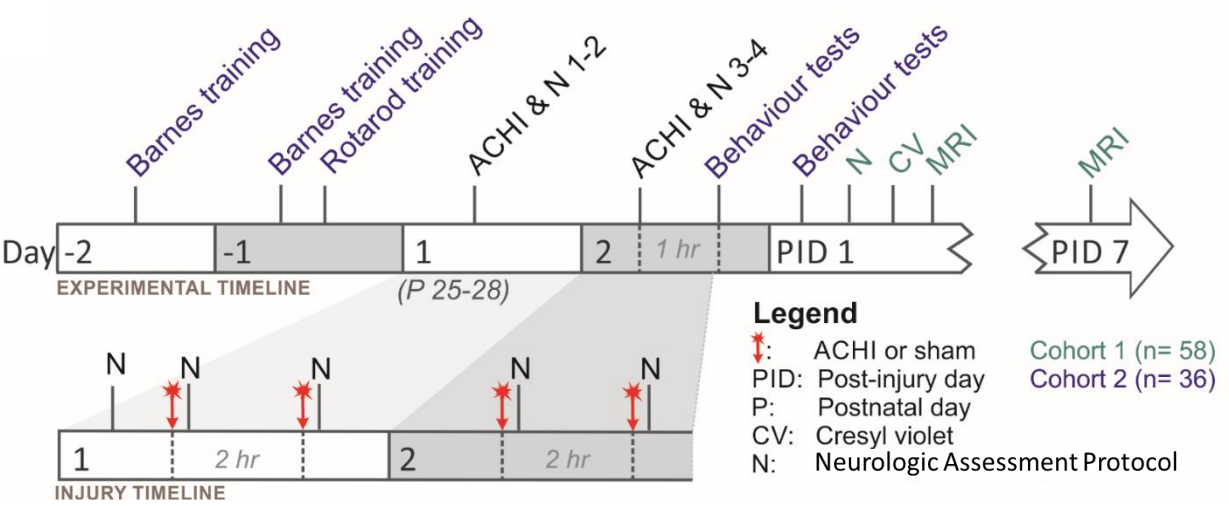


Figure 3.1: Experimental timeline. Two cohorts of male and female juvenile Long Evans rats underwent two separate sets of experiments. Animals in cohort 1 were used for neurologic severity scoring, MRI analysis, and cresyl violet histology. Animals in cohort 2 were trained for the Barnes maze and Rotarod in the two days immediately before ACHI, and then were tested in the Barnes maze, Rotarod, elevated plus maze, and open field maze at one hour and one day after the final ACHI. Both cohorts followed the same injury timeline: Animals in the repeat ACHI group received two impacts per day, with a two-hour interval between injuries, for two days. The single ACHI group received three sham procedures followed by one impact, on the same timeline as the repeat ACHI group. Sham control animals received four sham procedures on the same timeline as the other groups. All subjects were tested for consciousness and NAP immediately after each ACHI or sham, and a subset from cohort 1 were tested again 24 hours later.

days. The single ACHI group received three sham procedures followed by one impact, on the same timeline as the repeat ACHI group. Sham control animals received four sham procedures on the same timeline as the other groups. All rats were tested for consciousness and NAP immediately after each ACHI or sham, and a subset from cohort 1 were tested again 24 hours later.

3.2.3 Rat Welfare Monitoring

Restraint Tolerance

During all ACHI or sham procedures animals were assessed for tolerance of the restraint cone. Animals were scored on a scale of 0-4 (**Table 3.1**) based on willingness to enter the cone, and on movement and vocalisation while restrained. Any animal that reached a score of 4 or

Table 3.1: Restraint scoring during ACHI. The following scoring criteria are to be used by experimenters implementing the ACHI procedur in order to monitor rats' tolerance to restraint during the procedure

Score	Response to restraint	Researcher action
0	• No problem	• Proceed with impact
1	• Turn around before restraint is secured 1-2 times* • Little/no vocalisation (1-2 s) • Little/no squirming (1-2 s)	• Proceed with impact
2	• Turn around 3-5 times • Little vocalisation (3-5 s) • Little/no squirming (3-5 s)	• Proceed with impact
3	• Turn around 5-10 times • Some vocalisation (5-10 s) • Some squirming (5-10 s)	• Attempt to calm subject • Proceed with impact when possible
4	• Turn around >10 times • Frequent vocalisation (>10) • Frequent squirming (>10) • Continuous vocalisation/squirming • Tied in bag >5 minutes	• Return to home cage • Retry after a minimum of 15 minutes • Retry up to three times • Use food reward to encourage into bag
5	• Moved during impact – no injury	• Use as sham where possible
6	• Moved during impact – injury in wrong location	• If injury is not near correct location and data are outliers, remove animal from study

greater (e.g. >10 instances of vocalisation or movement; restrained in cone for 5 minutes) was immediately removed from the restraint and returned to the home cage for 15 minutes minimum. Protocol required that the procedure be re-attempted up to three times, and if it could not be completed the animal be removed from the study, however no animals were removed from the current study.

Pain Assessment

All animals were assessed immediately before, and for several days after, each ACHI for indications of pain or discomfort that included changes in locomotion (i.e. immobility, slowness of movement), behaviour (i.e. hunched posture, piloerection, excessive stretching, teeth grinding) pain on palpitation of the impact site (i.e. vocalization, withdrawal of head), skin turgor (i.e. increased tenting), and changes in weight relative to age and sex-matched littermates (decreased by more than 5%). **Table 2.2 shows** scoring scales developed with our institutional

Table 3.2: Pain scale and monitoring checklist after ACHI. The following criteria can be used in the days and weeks following ACHI to monitor rats level of discomfort and decide how to proceed if significant signs of distress appear

Criteria/Score	0	1	2	3
Locomotion	Moving normally around cage	Slow to move or hugging sides of cage	Reluctant to move -when stimulated	Moribund/ immobile
Behaviour	Animal calm in cage. Previously social animal still social	Previously social animal has become withdrawn or aggressive	Hunched posture, or piloerection, or excessive stretching and teeth grinding	Increased respiratory rate or labored breathing
Pain on palpitation of impact site	None	Mild (vocalizes quietly once when handled, flinches when site touched)	Moderate (vocalizes quietly more than once OR loudly once or twice when handled, and tries to escape)	Severe (loud and insistent vocalization, withdraws head, bites, struggles)
Skin Turgor	Normal			Reduced turgor (Skin tenting ≥ 2s)
Weight (relative to age & sex-matched litter mate average)	≥95%	90-94.99%	85-89.99%	<85%

Pain Scale Interpretation		
Highest Single Category	**Combined Score**	**Action**
0-1	**0-2**	NO supportive care Increase monitoring • Complete pain score monitoring checklist • Check every 4-6 hours during the day, max 12 hour interval overnight Improvements in condition • If pain score returns to 0 (s & c) return to cage-side monitoring OK to proceed with repeated impacts
2	**3-5**	Provide supportive care where appropriate e.g.: • Food on floor • Soak food • Administer fluids • Heat source Increase monitoring • Complete pain score monitoring checklist • Every 4-6 hours Improvements in condition • If pain score recovers to 0-1 (s) or 0-2 (c) within 12 hours continue supportive care for additional 6 hours ○ If recovery is maintained return to normal cage-side monitoring • If pain score does not recover to 0-1 (s) or 0-2 (c) within 12 hours of supportive care, remove and euthanize animal Do NOT proceed with repeated impacts
3	**≥6**	Contact principle investigator, lab personnel, or veterinarian to confirm Euthanize animal

Take action based on greatest score. e.g. An animal scoring 1 in each category has a highest single category score of 1, and a combined score of 4, and should therefore begin supportive care and increased monitoring

Animal Care Committee. Animals were rated in each of the 5 categories on a scale of 0 (normal) to 3 (severe) with a score of greater than 2 in any category, or a combined score of greater than 3, requiring the animal be given supportive care. Animals with a score of 3 in any category or a combined score of greater than 6 were to be removed from the study and euthanized.

3.2.4 MRI Acquisition & Analysis

To further examine structural damage in the ACHI model we performed *ex vivo* structural MRI on both male and female cohorts of sham, single- or repeated ACHI rats at one and seven days after injury. The number of brains scanned for each of the groups were: sham, 1 day = 8 (3 males, 5 females); sham, 7 days = 9 (4 males, 5 females); single mTBI, 1 day = 11 (4 males, 7 females); single mTBI, 7 days = 7 (5 males, 2 females); repeated mTBI, 1 day = 11 (5 males, 6 females); repeated mTBI, 7 days = 7 (3 males, 4 females). Animals were perfused as described above, and after the brains were removed, they were embedded in agar gel (Webster et al., 2015) and scanned using a 4.7 Tesla Bruker Advance III MRI fitted with a BGA12S2 actively shielded gradient set. Actively decoupled volume transmit and 4-channel surface receive coils (Bruker, Germany) were used to acquire a multi-echo, T_2^*-weighted image with the following imaging parameters: repetition time = 8 s; 12 echoes with the first echo at 15 ms and an echo spacing of 7.5 ms; field of view = 2.304 × 2.048 mm^2; matrix size = 144 × 128; resolution = 160 × 160 μm^3; number of slices = 74; slice thickness = 160 μm; and number of excitations = 2.

Spatial intensity in homogeneity in T_2^*-weighted images was corrected by estimating the bias field with N4 Bias Correction (Tustison et al., 2010) and template images generated for sham, single and repeated injury cohorts at each time point using Advanced Normalization Tools (ANTs, http://stnava.github.io/ANTs/) (Avants et al., 2011). The resulting template images

were then combined using ANTs into a study-specific template (D. K. Wright, Liu, et al., 2016; D. K. Wright, Trezise, et al., 2016) that was segmented into different tissue classes using FAST (Y. Zhang, Brady, & Smith, 2001). The FAST segmentations were used to guide the tracing of six *a-priori* regions of interest (ROIs) including the ipsilateral and contralateral cortex, corpus callosum and hippocampus (Johnstone et al., 2015; X. L. Tan et al., 2016; Webster et al., 2015). The ROIs were registered to rat space using inverse rat-to-template diffeomorphisms and the total volumes for each structure were calculated using FSL stats, a component of FMRIB's Software Library (FSL, www.fmrib.ox.ac.uk/fsl). MRI analysis was conducted by a researcher who was blinded to the experimental conditions.

3.2.5 Behavioral Assessment

In addition to the NAP, four behavioural tasks were employed to examine the behavioral sequelae to repeated ACHI in both male and female animals. A cohort of repeated ACHI (n= 9 female, 9 male) and sham injured rats (n= 9 female, 9 male) were assessed using the following behavioural tasks at one hour and one day after final injury:

Barnes Maze

The Barnes maze was used to assess spatial memory after injury. The Barnes maze (Maze Engineers, Cambridge, MA) is an elevated circular platform (122 cm diameter) with 20 holes (10cm diameter) spaced evenly around the perimeter. One hole leads to an escape box that remains in the same position throughout the experiment, and the other holes are open. The maze was placed in a room with distinct distal visual cues to allow for spatial navigation. The training paradigm used was adapted from common procedures in order to suit the timeline of this experiment and the juvenile age group (Rosenfeld & Ferguson, 2014). All rats were trained

to locate the escape hole for two days before ACHI or sham. The first training day consisted of 4 trials, and on the second training day trials repeated until criterion was reached. The training criterion was determined as the ability to locate the escape hole using a direct search method with ≤ 1 error in two trials. Immediately before the first training trial rats were enclosed in the escape box to acclimatize for 2 minutes. Then they were moved directly to the center of the maze and allowed to explore freely for 5 minutes. If they found the escape box during this time, they remained there for 15 seconds before being returned to their home cage. If they did not find the escape box, they were led there by the researcher and allowed to remain there for 15s before being returned to their home cage. All remaining training trials were the same as the first, except the acclimatization period was not repeated, and instead rats were placed in the center of the maze directly from their home cage at the start of the trial. A test trial was completed one hour and one day after injury. Like the training trials, rats were placed in the center of the maze and allowed to explore freely for up to 5 minutes. The total distance travelled and number of errors made before locating the escape hole was measured as an indication of spatial memory. Movement in the maze was tracked using EthoVision XT 11.5 software (Noldus, Netherlands). Error tracking was performed manually by a researcher blinded to group. An error was scored if the rat moved any portion of their head over a hole that did not allow escape. All maze components were wiped down with Virkon disinfectant/cleaner and allowed to dry completely between rats.

Rotarod

Motor coordination and balance were assessed using the Rotarod (Rat Rotarod NG, Model 47750; Ugo Basile, Varese, Italy). The apparatus consists of a rotating rod (6 cm diameter)

with machined grips, divided into four equal 8.7 cm wide sections raised 30 cm above trip boxes. Rats were trained to use the Rotarod one day before the first ACHI or sham. In training trials, rats were placed on the rod, which was rotating at a constant speed of 10 rpm. The training trial continued until the rat able to stay on the rod for 60 consecutive seconds without falling, turning around, or clinging to the rod. If they fell from the rod or turned around, they were placed back on the rod correctly and the timer restarted. In test trials, an accelerating protocol was used with the speed of rotation increased from 10 – 50 rpm over 300 s. Each trial was terminated if an animal fell, clung and rotated for two full rotations, or remained on for >300s. Latency to fall (s) were automatically recorded for each trial. The average of the three trials was calculated and used for analysis. Training trials and baseline values were recorded 24 h prior to ACHI procedure. The Rotarod apparatus was wiped down with Virkon and allowed to dry completely between rats.

Open Field

The open field was used to assess anxiety-like behaviour, and overall locomotion. Animals were placed in the center of a circular white, arena (100 cm diameter, 50cm walls in a brightly lit room and given 5 min to explore freely (N. C. Jones, Cardamone, et al., 2008; Shultz et al., 2015; X. L. Tan et al., 2016). Animals were tracked with EthoVision XT 11.5 software (Noldus, Netherlands). Increased time spent in the perimeter (thigmotaxis) or decreased time spent in the center area (70 cm diameter) are measures of anxiety-like behaviour (N. C. Jones, Salzberg, et al., 2008; Prut & Belzung, 2003). Secondary measures included proportion of time moving and average velocity of movement. The maze was wiped down with Virkon and allowed to dry completely between rats.

Elevated Plus Maze

Anxiety like behaviour was also assessed in the elevated plus maze after ACHI (Brocardo et al., 2012; Hawley, Morch, Christie, & Leasure, 2012). A raised plus-shaped maze with two opposing enclosed arms and two opposing open arms in a brightly lit room was used. Rats were placed in the center of the maze facing a closed arm, and allowed to explore freely for 5 minutes. Animals were tracked using EthoVision XT 11.5 software (Noldus, Netherlands). The proportion of time spent in the open arms was measured as an indication of anxiety. Secondary measurements taken were proportion of time moving, and average velocity. The maze was wiped down with Virkon and allowed to dry completely between rats.

3.2.6 Statistical Analysis

A Kruskal-Wallis test with Nemenyi *post hoc* analysis were used to compare the composite NAP, as this is a non-parametric dataset. Two-way ANOVAs with injury group and post-injury time point as the between subject factors were used to analyze MRI volumetrics for each ROI. For Barnes maze training trials, mixed ANOVA with trial number as within subject factor and sex as between subjects factor was used to analyze individual trial path lengths. A Greenhouse-Geisser correction was used to adjust for violation of Mauchly's test of Sphericity. Two-way ANOVA with injury group and sex as between subject factors was used to analyze total path distance. Mixed ANOVA with injury group and sex as between subjects factors, and post injury time point as the within subjects factor were used to analyze behavioural task outcomes. Statistical significance was set at $p<0.05$. Linear trendlines and correlation coefficients in the comparison of NAP scores to Barnes maze outcomes were determined using Excel (Microsoft, Redmond, WA). Power analyses were performed using G*-Power to determine

group sizes required for appropriate statistical analyses. Statistical analyses were performed using RStudio (RStudio, Boston, MA) and SPSS statistics software (IBM, New York, NY).

3.3 Results

3.3.1 Rat welfare

The animals' welfare and comfort were prioritized by taking extra care to actively monitor and respond to changes in each animal's tolerance of the restraint, and signs of pain post injury.

Restraint tolerance

Animals were assessed for restraint tolerance according to a predetermined scoring index approved by our institutional Animal Care Committee. Overall the rats tolerated the restraint well. Common indications of low tolerance were mild vocalisation, and shifting position in the restraint. The ACHI was only performed if the rat was motionless for the impact component and was not vocalizing during the procedure. In the current cohort (n=97), there were no instances where a rat had to be removed from the study due to restraint intolerance. There was one instance (repeated ACHI male, 4th injury) where the rat was returned to their home cage due to persistent movement, but the procedure was successful on the second attempt.

Pain assessment

Rats were regularly assessed for signs of pain, and scored according to a predetermined index throughout the experiment and following days. Rats showed almost no signs of pain across all criteria, and no animals received a combined pain scale score of greater than 2, so supportive care and/or analgesics were not required for any rats in these test populations.

Mortality

The overall mortality rate for this model was relatively low at 2.5% (3/119). These cases were all males from the repeated ACHI group, following their fourth ACHI procedure. In all three cases, it was immediately obvious that the rat was moribund, so they were immediately euthanized and removed from the study. There were no cases where a rat initially appeared normal, and then escalated to a moribund state.

These data indicate that the ACHI procedure is a simple and high throughput method of producing a mild closed head injury. The ACHI procedure was consistently completed, including the NAP, in less than 5 minutes per animal.

3.3.2 Neurologic impairment and loss of consciousness after ACHI

Loss of Consciousness

We were able to assess three indicators of loss of consciousness (LOC) (apnea, toe pinch reflex, and righting reflex) immediately after each ACHI or sham procedure, without the potential confound of recovery from anaesthesia. As expected, rats in the sham group did not exhibit any indication of LOC for any of the three indicators. Apnea was not observed following the ACHI procedure in any of the 93 rats in any of the conditions (sham, single, repeat). The toe pinch reflex was absent in one animal from the repeated ACHI group for 5 seconds. The righting reflex was briefly impaired in 11% of single ACHI animals and 20% of repeated ACHI rats (**Fig. 3.2A**). For these rats, the average latency until the righting reflex recovered was 4 seconds in the single ACHI group and 21.75 seconds in the repeated ACHI group. Chi square analysis indicate there are no significant differences in the proportion of animals from each group that failed toe-pinch (χ^2 (2, N=58) =1.93, p=0.38) and righting reflex tests (χ^2 (2, N=58) =4.33, p=0.11).

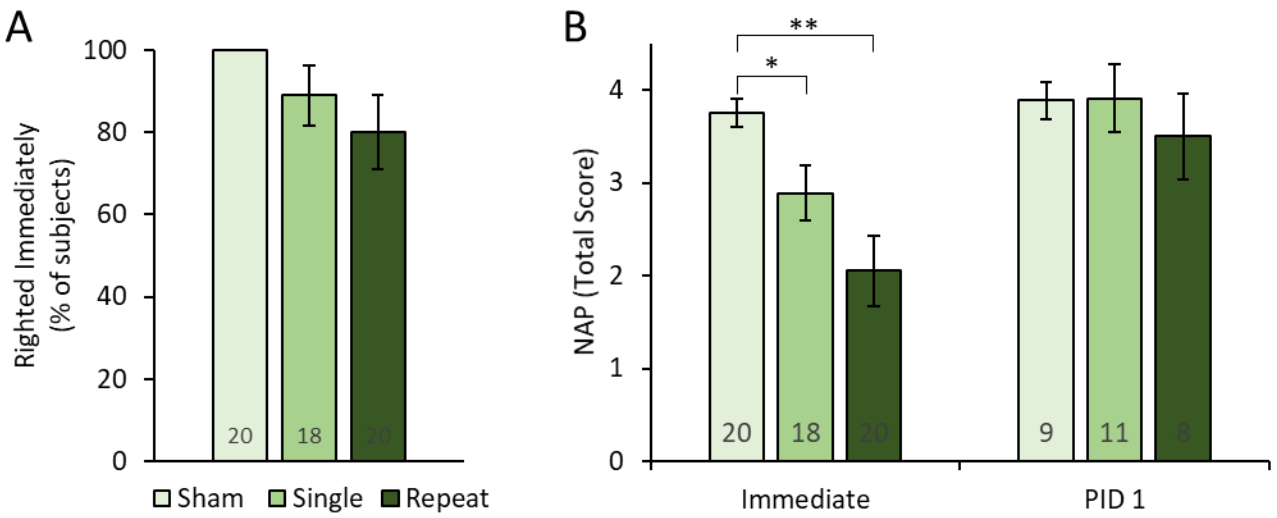


Figure 3.2: ACHI caused short-lived loss of consciousness and acute neurologic impairment. (A) Righting reflex was assessed immediately after ACHI or sham. 100% of sham subjects self-righted immediately, whereas only 89% of single ACHI, and 80% of repeat ACHI rats self-righted immediately. This delay to self-right indicated a short-lived loss of consciousness. **(B)** NAP testing began immediately after the righting reflex test, and was repeated one day later in a subset of animals. They were assessed in the startle reflex, limb extension, beam walk, and rotating beam tasks, and given a point for each successfully completed task so that a score of 4 indicates perfect performance, and a score of 0 indicates severe impairment. Immediately after injury or sham the average NAP was significantly lower in single and repeat ACHI groups compared to sham. 1 day after ACHI or sham there were no significant differences in NAP. (* $p<0.05$; ** $p<0.01$ | ACHI awake closed head injury; NAP neurologic assessment protocol)

Neurological Assessment Protocol

NAP score analysis was completed in a cohort of 58 rats immediately after each ACHI or sham (n=18-20 per group), and in a subset of these it was repeated 24 hours later (n=8-11 per group). NAP tasks successfully completed by each animal was quantified for each group and presented as a composite score, where a score of 4 indicates perfect performance and 0 indicates severe impairment. Immediately after the procedure, average NAP scores for the sham, single ACHI, and repeated ACHI groups were 3.8, 2.9, and 2.1 out of 4 respectively (**Fig. 3.2B).** Kruskal-Wallis analysis revealed a significant effect of injury group (χ^2 (2) =19.35, p=0.000063),

and Nemenyi *post hoc* analysis showed that both single ($p<0.05$) and repeated ACHI ($p<0.001$) groups had significantly lower scores than sham animals. One day after the final ACHI, there were no significant differences between any of the treatment groups in NAP scores (χ^2 (2) =5.25, p=0.07).

Since this is a new condensed format of components routinely performed in other common neurological assessments(Hsieh et al., 2017; Anthony L. Petraglia et al., 2014; Schaar et al., 2010; Shapira et al., 1988) we also assessed each task individually in order to determine how effectively they discriminate between injury groups. The success rates for each task (i.e. the percentage of rats in each group to pass each task) are shown in **Figure 3.3**. In the startle response and limb extension tasks, 100% of shams passed, whereas only 55% and 40% of repeat injured rats passed, respectively. The beam walk and rotating beam tasks appeared to be more innately challenging, since a subset of sham rats failed this task as well. The beam walk still

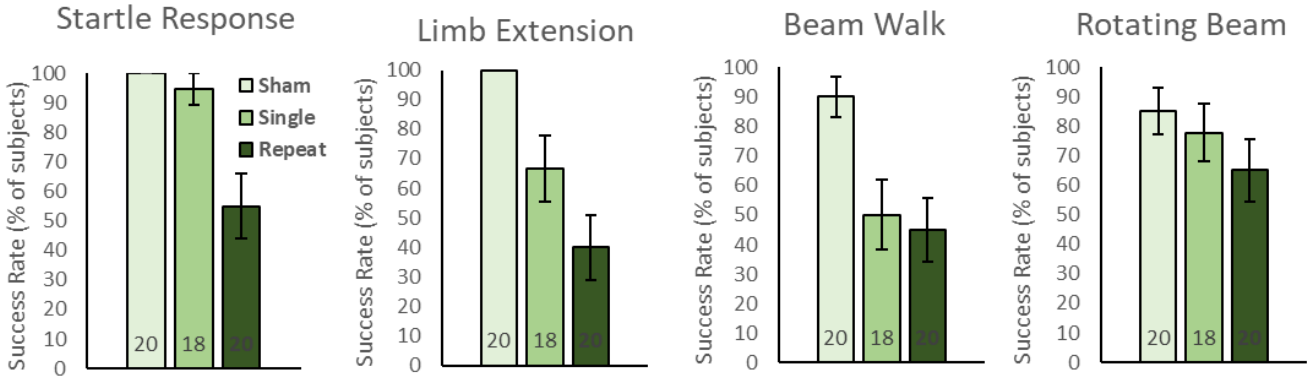


Figure 3.3: Comparison of success rate for each NAP component. The four tasks used in our modified NAP can individually distinguish between injury groups with varying success. Startle response was lost in one single injured subject, and in 45% of repeat injured rats. Limb extension was impaired in 33% of single injured rats, and in 60% of repeat injured rats. Beam walk performance was impaired in 55% of repeat injured and 50% of single injured animals, but may have been more challenging overall as 10% of shams also failed. Similarly, 15% of sham controls failed to complete the rotating beam task, however injury-related impairment was evident as 22% of single- and 35% of repeat injured rats also failed. Error bars show standard error of the proportion. (NAP neurologic assessmet protocol).

appeared to differentiate between experimental groups, since 90% of the sham group passed, compared to 45% of repeat injured rats. The rotating beam task may be less effective for differentiating between groups, since only 85% of the sham group passed compared to 65% of repeat injured rats.

3.3.3 No volumetric changes in structural MRI after ACHI

Significant structural damage is unusual in clinical cases of concussion(McCrory et al., 2013), but common in many rodent models of traumatic brain injury where the brain is directly impacted through a craniectomized skull (e.g.,(D. K. Wright, Trezise, et al., 2016)). Structural MRI was performed on a separate cohort of animals to determine if the ACHI procedure led to volumetric changes in several regions of interest. As shown in **Figure 3.4A**, no significant regions of damage could be identified with the structural MRI performed on the brains of animals that received either single or repeated ACHI. This observation was supported by the lack of a significant injury or recovery time effect or interaction on volumetric measures from the six ROIs (**Fig. 3.4B)**.

3.3.4 More errors were made in the Barnes maze probe after 4xACHI

Memory deficits are a common cognitive symptom of concussion in clinical populations (McCrory et al., 2013) and rodent models alike(Luo et al., 2017; A L Petraglia et al., 2014; Shultz et al., 2012; D. K. Wright, Trezise, et al., 2016), and in these experiments we used the Barnes maze to determine whether our ACHI model impairs recall memory. Rats were trained to locate a small escape hole on an open elevated circular platform using distal spatial cues (n=9 per group) prior to undergoing the ACHI procedure. After the ACHI procedure, their ability to

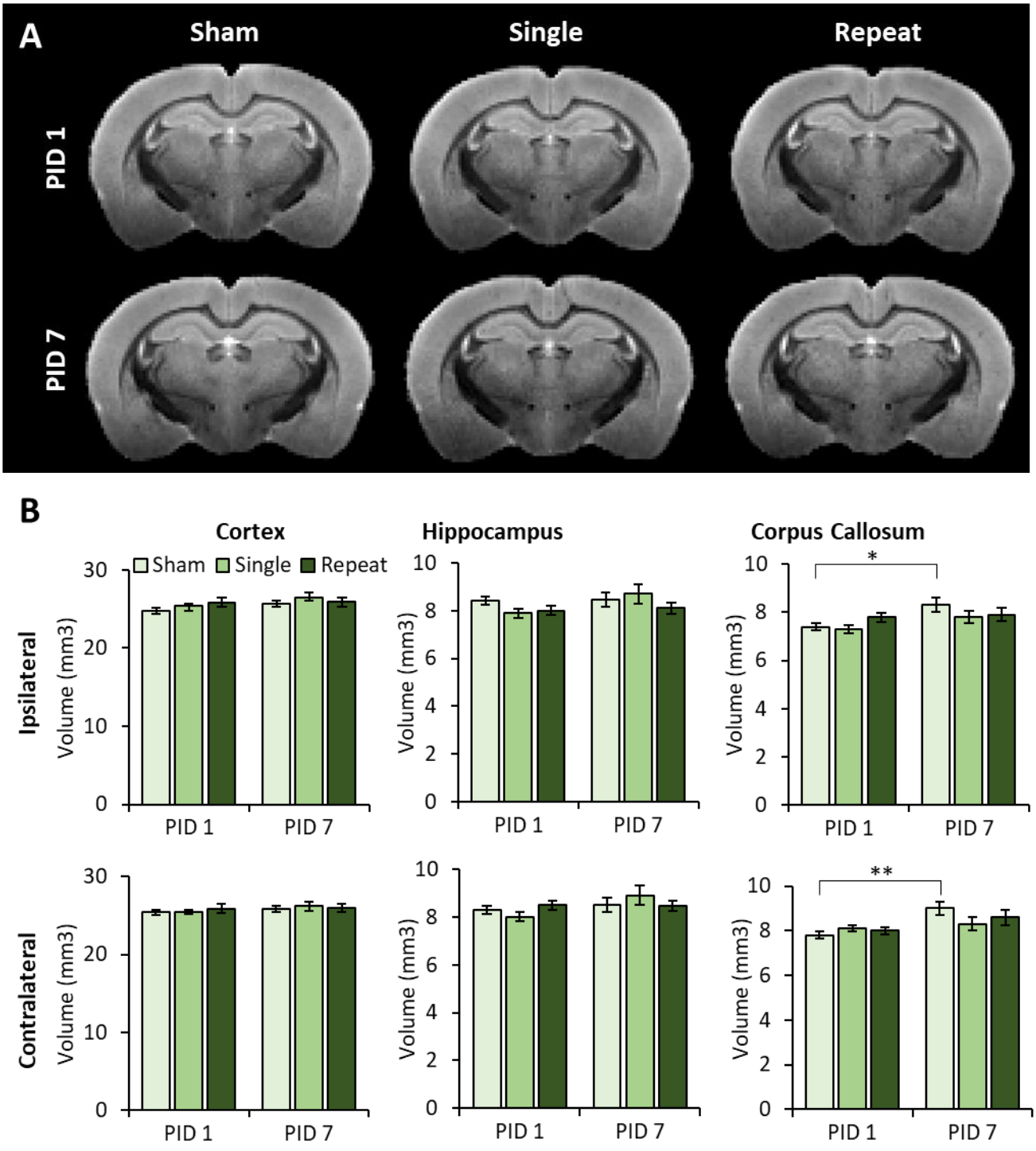
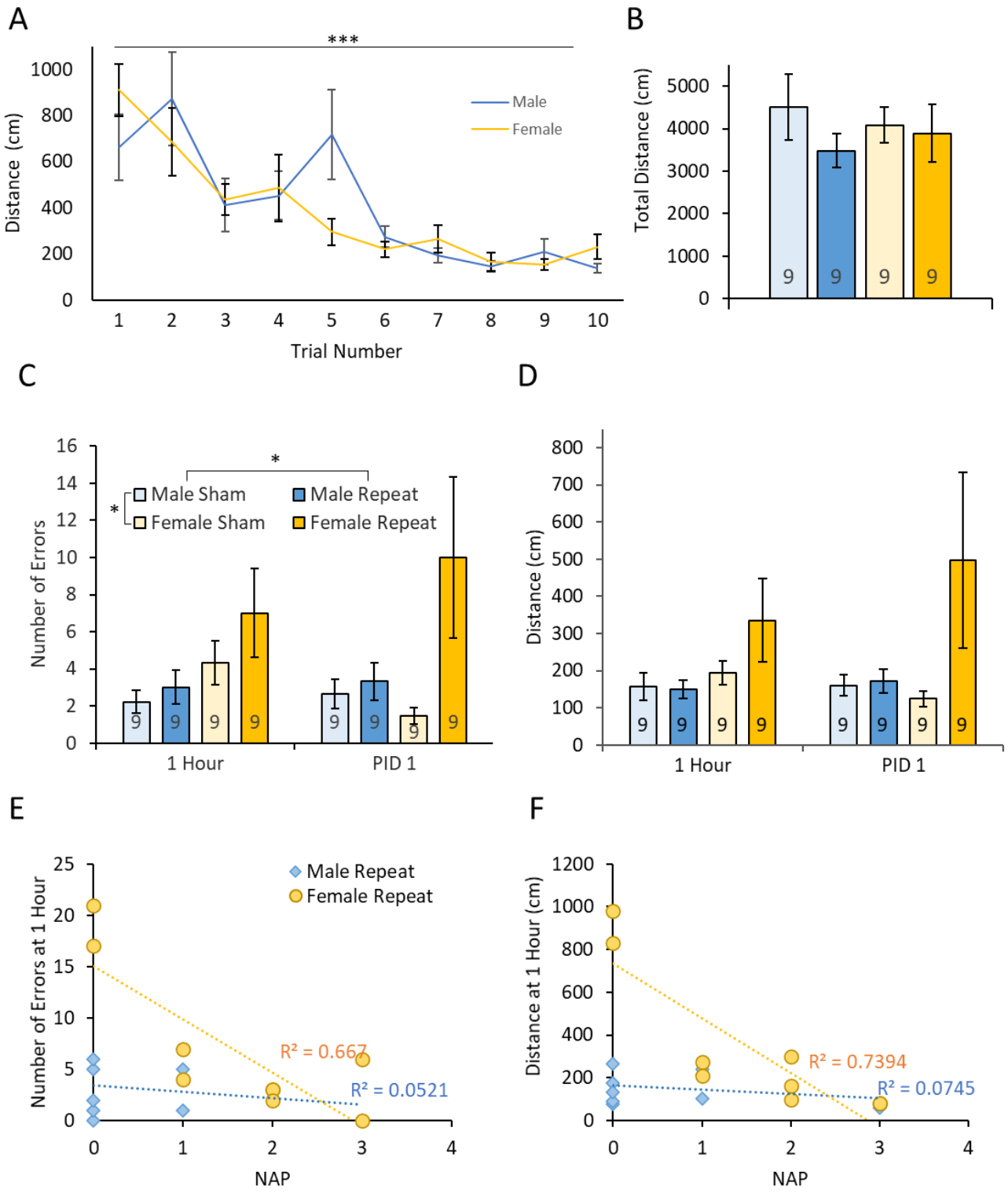


Figure 3.4 ACHI does not produce structural MRI abnormalities. Single and repeat ACHI did not lead to overt brain damage observed in ex vivo structural MRIs in juvenile male and female rats. **(A)** Representative coronal slices of MRI scans at one and seven days following sham, single, or repeat ACHI show no obvious damage. **(B)** The volume of the ipsilateral and contralateral cortex, hippocampus, and corpus callosum were measured at PID1 or PID7. There was no significant effect of injury, or injury and recovery interaction in each of the six regions of interest. There was a significant effect for recovery time on volumes of the ipsilateral and contralateral corpus callosum. (* $p<0.5$; ** $p<.01$| ACHI awake closed head injury; MRI magnetic resonance imaging; PID post injury day)

remember the location of the escape hole was assessed. There was a significant effect of time ($F(4.402, 149.680) = 14.681$, $p<0.001$) but no interaction with sex across training trails, indicating that all rats were able to learn the location of the escape hole (**Fig. 3.5A**). On average this required 10 training trials). After randomly assigning rats to injury groups, no differences were found in the total distance travelled during training (**Fig. 3.5B**) confirming that groups were equally proficient in the task before injury.

The total number of errors, and total distance taken to locate the Barnes maze escape hole were measured one hour and one day after ACHI. As shown in **Figure 3.5C**, a significant main effect of injury group ($F(1, 32) = 5.857$, $p=0.021$), indicated that across both sexes and both time points, the animals from the repeated ACHI group made more errors during testing. A significant main effect of sex ($F(1, 32) = 4.874$, $p=0.035$), indicates that across injury groups and time points, females made more errors than males. There were no significant effects of time point, or interactions between factors. As shown in **Figure 3.5D** There were no significant interactions or effects on the total distance to locate the escape hole, ($F(1, 32) = 4.156$, **$p=0.050$**) with a moderate effect size ($\eta_p^2 = 0.115$), indicating further exploration is warranted.

In order to determine if a subset of rats are more impaired than most, the number of errors made (**Fig 3.5E**) and distance to escape (**Fig 3.5F**) were plotted individually against NAP scores for repeated ACHI rats. This also allowed us to determine whether NAP scores may predict cognitive outcomes. In the female repeated ACHI group, moderate negative correlation was observed between the number of errors made and NAP score ($\mathbf{R^2} = 0.667$), and the distance travelled to escape ($\mathbf{R^2} = 0.739$). This was not present in the males, and had subsided in the females at PID 1 ($\mathbf{R^2} < 0.1$).


A
***
Distance (cm)
1000
800
600
400
200
0
1 2 3 4 5 6 7 8 9 10
Trial Number
Male
Female
B
Total Distance (cm)
5000
4000
3000
2000
1000
0
9 9 9 9
C
*
*
Male Sham
Male Repeat
Female Sham
Female Repeat
Number of Errors
16
14
12
10
8
6
4
2
0
9 9 9 9 9 9 9 9
1 Hour
PID 1
D
Distance (cm)
800
700
600
500
400
300
200
100
0
9 9 9 9 9 9 9 9
1 Hour
PID 1
E
Male Repeat
Female Repeat
Number of Errors at 1 Hour
25
20
15
10
5
0
R² = 0.667
R² = 0.0521
0 1 2 3 4
NAP
F
Distance at 1 Hour (cm)
1200
1000
800
600
400
200
0
R² = 0.7394
R² = 0.0745
0 1 2 3 4
NAP

Fig**ure 3.5: Repeat ACHI produced mild acute cognitive deficits in the Barnes maze**. **(A)** Rats were trained to use distal spatial cues to locate the escape hole in a Barnes maze in a series of 5 minute training trials over two days. The predetermined training criterion was to locate the hole using a direct search strategy with 1 or fewer errors in two trials. Rats were able to reach the training criterion within an average of ten trials. There was a significant effect of trial number, but no significant interaction with sex. **(B)** The total combined distance travelled during all training trials was equivalent between groups, indicating that all groups were equally proficient in the task before ACHI or sham. **(C)** There was a significant main effect of injury, indicating the 4xACHI group made more errors regardless of sex or time point. There were no significant interactions of injury, sex, or post injury time point. Similarly, a significant effect of sex indicates that across injury groups, females made more errors than males. **(D)** There were no significant differences in the total distance travelled to escape during test trials**. (E-F)** The number of errors and distance to escape versus NAP for the 4xACHI group, in order to determine whether NAP performance predicted cognitive outcomes. **(E)** In females only, a moderate negative correlation was observed between NAP and errors (R2 = 0.667); and (**F)** between NAP and distance taken to escape (R2 = 0.739) the Barnes maze at one hour. (* $p<0.05$, *** $p<0.001$ | PID post-injury day)

3.3.5 Anxiety and motor performance were not affected by ACHI

We used the Rotarod apparatus to examine learned motor skills after either single or repeated ACHI (n=9 per group). All rats were trained to the same criterion on the Rotarod prior to ACHI, and then tested after injury. The test protocol accelerated from a speed of 10 to 50 RPM in 5 minutes, and the latency to fall was recorded. As shown in (**Figure 3.6A**), there were no significant group, sex, or time point effects or interactions in Rotarod performance, indicating no motor impairment.

The total distance travelled by animals in an open field maze can also be used as a convergent means to assess motor function (**Fig. 3.6B**). As is shown, there was no effect of ACHI on the total distance travelled in any group, providing further evidence that mobility was not impaired. Further support comes from the fact that the average speed and percentage of time spent moving in the Barnes maze, open field, end elevated plus maze was equivalent across groups (**Fig. 3.7**). In all mazes, there were no significant injury effects or interactions on the average speed or time spent moving, confirming the absence of ACHI-induced motor impairment. Conversely, in the Barnes maze there was a significant effect of sex ($F(1,32) = 4.296$,

p<0.046) on average speed, indicating that the females were moving significantly faster on both days, regardless of injury group.

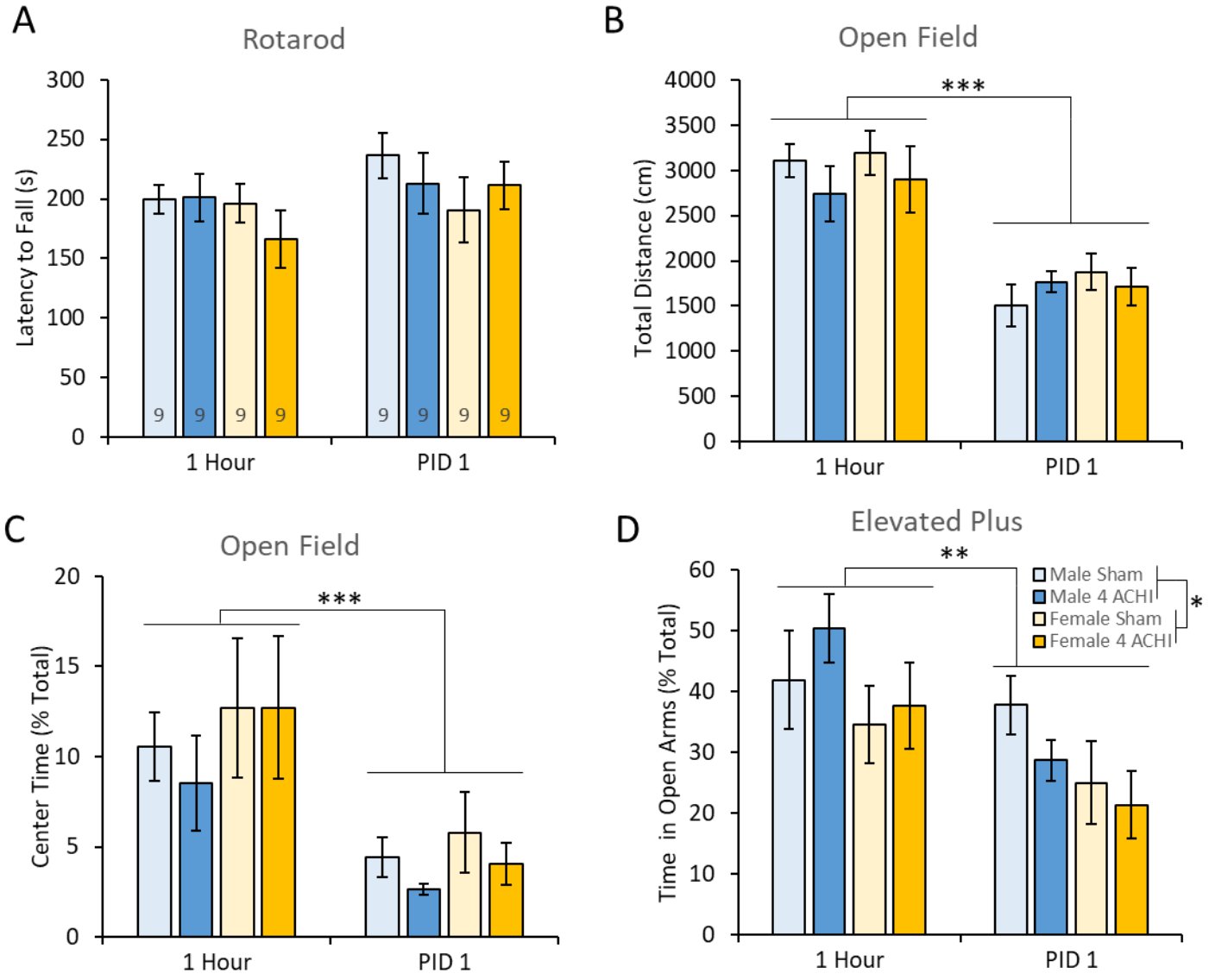


Figure 3.6: Repeat ACHI did not produce significant motor impairments or anxiety like behaviour. (A) There was no significant effect of injury, sex, or timepoint on the latency to fall from the Rotarod. Rats were trained to use the Rotarod prior to injury, and then tested on a protocol accelerating from 10 to 50 RPM in a 5 minute interval. **(B)** Rats were placed in a 100 cm diameter open field maze and allowed to explore freely for 5 minutes. Repeat ACHI did not impair overall mobility, as there were no significant differences in the total distance moved. A significant effect of post injury time point indicates all groups and sexes travelled a shorter distance on PID one. **(B)** There was no significant effect of sex or injury on the average proportion of time time spent in the center (70cm diameter) of the open field maze, but all rats spent significantly less time in the center on PID 1. **(D)** There was a significant effect of sex on the amount of time spent in the open arms of the elevated plus maze, indicating that across groups and time points females spent less time in the open arms. (* p<0.05; ** p<0.01; *** p<0.001 | PID post-injury day | n shown at base of graph in A were also used in B,C,D).

The amount of time spent in the center (70cm dia) of a round open field maze, and in the open arms of an elevated plus maze were used to examine signs of anxiety after ACHI (n=9 per group). In the open field there were no significant injury group effects, sex effects, or interactions for the amount of time spent in the center (**Fig. 3.6C**). Similarly, there were no significant injury group effects on time spent in the open arms in the elevated plus maze, indicating ACHI did not lead to acute elevations in anxiety (**Fig. 3.6D**). There was a significant effect of sex ($F(1,32) = 4.976$, $p=0.021$) meaning that females in all groups spent significantly less time in the open arms on both days, which is a putative indicator of anxiety. Conversely, the

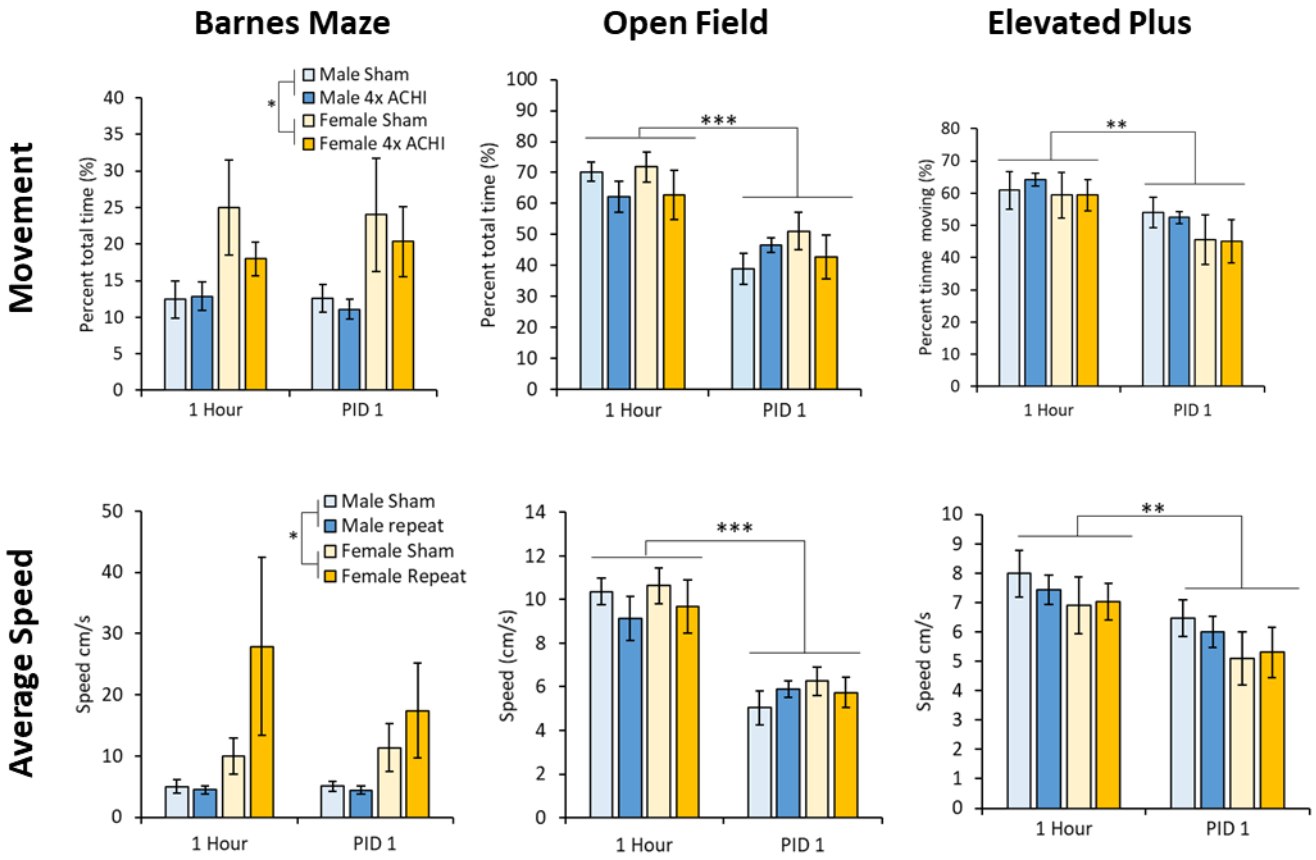


Figure 3.7: Speed and movement in behaviour mazes. The average speed, and proportion of time moving in the Barnes, open field, and elevated plus mazes were recorded as secondary measures of general locomotion. There was a significant effect of sex on both speed, and percentage of time moving in the Barnes maze. There were no significant effects or interactions of group or sex in the open field and elevated plus maze. All groups travelled significantly slower and spent significantly less time moving in the open field on PID1. (* $p<0.05$, ** $p< 0.01$; *** $p<0.001$ PID post-injury day; ACHI awake closed head injury)

significant effect of time on open field distance ($F(1, 32) = 102.577$, $p<0.001$); open field speed ($F(1, 32) = 102.577$, $p<0.001$); open field time moving ($F(1, 32) = 102.577$, $p<0.001$); and elevated plus maze time moving ($F(1, 32) = 102.577$, $p<0.001$), provided evidence of normal changes in performance following multiple exposures to these mazes.

3.4 Chapter Summary and Conclusions

We set out to design and characterize a model that produces mild closed head injury in juvenile rats that it is relevant to pediatric concussion. Taken together these observations reflect all four of the criteria that define a concussion outlined by the most recent consensus statement on concussion in sport (McCrory et al., 2013). We have shown that 1) an "impulsive" force transmitted to the head results in 2) the rapid onset of short-lived neurologic impairment that resolves spontaneously. This occurs 3) in the absence of overt structural changes, and 4) involves LOC and cognitive impairment in a small subset of cases. These results demonstrate the utility of the ACHI model for continuing preclinical research on concussions in the developing brain. Future studies should use a more challenging behavioural context to identify more subtle cognitive impairments. They should also use histological and advanced MRI approaches to identify microscopic damage not visible on structural MRI.

Chapter 4 – Repeated ACHI caused diffuse neurodegeneration and impaired cognitive flexibility in the first week of recovery

The following experiments are summarized in a complete manuscript that has been prepared for submission, but not yet submitted as of March 1, 2021.

Contributions:

A.M was responsible for all experimental design, ACHI induction, behavioural experiments, histology design and optimisation, some histological processing, statistical analysis, writing, and figure preparation. Erin McDonagh assisted with histological staining, imaging, and image processing in partial fulfillment of the requirements of her Honours thesis.

4.1 Chapter Abstract

Concussions are closed-head injuries that can result from transmission of biomechanical force to the brain. Symptoms vary greatly between individuals, and can include acute neurologic dysfunction, cognitive impairment, and executive dysfunction, amongst numerous others. Although concussions are categorized as a *mild* traumatic brain injury, symptoms can be severe and disruptive to daily life. Preclinical animal models that replicate the effects of human head injuries in laboratory animals are an essential tool for investigating concussion pathophysiology. This is an important step to identify new treatments and diagnostic strategies. A variety of models are needed to address the vastly heterogeneous etiology and symptomology of these injuries. We developed a new preclinical concussion model for juvenile rats, which does not require anaesthesia. In a previous characterisation we found repeated injury produced acute neurologic impairment in the NAP, and mild memory impairment in Barnes maze acquisition, in the absence of structural MRI abnormalities. A subsequent study in this cohort found white matter abnormalities using in diffusion weighted MRI (Wortman et al., 2018). Continued characterisation is needed to identify potential sources of these white matter abnormalities, and to demonstrate additional clinically relevant behavioural outcomes. We hypothesized that axonal degeneration contributes to white matter abnormalities observed after repeat injury, and that the Barnes maze reversal task would reveal more subtle cognitive deficits. To address this, juvenile Long Evans rats were given eight awake closed head injuries in two days. Our neurologic assessment protocol was used to test neurologic function immediately after each injury. The Barnes maze and reversal task were used to detect cognitive impairment in the first three days of recovery. FD NeuroSilver™ II histology was used to

identify neurodegeneration in several regions of interest on post-injury day three and seven. We found that repeated awake closed head injury produced acute neurologic deficits in the neurologic assessment protocol. A mild impairment to spatial learning mediated by impaired cognitive flexibility was observed in Barnes maze reversal training on post-injury day two. Diffuse neurodegeneration was observed in the optic tract and hippocampus on post-injury days three and seven, and in the cortex on post-injury day seven. These are analogous to clinical trends, further establishing ACHI as a clinically relevant model of pediatric concussion.

4.2 Materials and Methods

4.2.1 Subjects

A total of 16 female and 16 male juvenile Long Evans rats were used for these experiments. The Long Evans strain was selected because they perform better in cognitive behavioural tasks (Turner & Burne, 2014).They were purchased from Charles River Laboratories (Montreal, QC) and transported to the University of Victoria on postnatal day (PND) 10-14. They were transported and housed with their dam in litters of 10. On PND 21 they were weaned, and housed in same-sex groups of two to three littermates. They were pseudo-randomly split into the repeated injury group or cage control group, with sexes divided evenly into each. They were housed so that each cage held rats from both the control and repeated injury groups. All procedures were approved by the University of Victoria's Animal Care Committee, and followed the Canadian Council for Animal Care's guidelines. **Figure 4.1** summarises the group breakdown and experimental timeline.

4.2.2 Experimental Timeline

The ACHI model was used to induce repeated, helmeted, closed head injuries over the left parietal cortex without anaesthesia (Meconi et al., 2018). Beginning on PND 25-26, rats underwent the ACHI procedure as we have previously described (Christie et al., 2019a). Briefly, rats were loosely restrained in a soft plastic restraint cone. A 3D-printed plastic helmet was positioned, with the impact surface centered over the left parietal cortex. The rat was placed on a soft foam pad, and positioned under a Leica Impact One electromagnetic piston (Leica Biosystems, Buffalo Grove, IL) modified with a rubber impact tip (5mm diameter). An impact with a displacement of 10mm at a speed of 5m/s and a dwell time of 0.1 seconds was delivered to the impact surface on the helmet. The rat was immediately removed from the restraint in order to monitor the recovery. Rats in the repeated injury (8xACHI) group received 4 injuries per day for two days, with a two hour interval between each injury. LOC was determined by observing rats for apnea, delayed toe pinch reflex, and delayed righting reflex as described previously (Meconi et al., 2018). Cage control rats underwent the LOC tests on the same timeline.

4.2.3 Neurologic Assessment Protocol Scoring Update

In order to measure acute neurologic impairment, rats underwent our previously established NAP immediately after LOC tests (Christie et al., 2019a; Meconi et al., 2018). The NAP consists of four tasks that assess reflex and motor function. It can be administered rapidly within a minute after injury, and has an analogous purpose to rapid clinical assessment tools like the cSCAT5 (Davis et al., 2017), SCAT5 (Petit, Savage, Bretzin, Anderson, & Covassin, 2020), and CRT (Echemendia, Meeuwisse, McCrory, Davis, Putukian, Leddy, Makdissi, Sullivan,

Broglio, Raftery, Schneider, Kissick, McCrea, Dvorak, Sills, Aubry, Engebretsen, Lossemore, Fuller, Kutcher, Ellenbogen, Guskiewicz, Patricios, & Herring, 2017). Each of the four NAP tasks is scored on a scale of 0-3 and then they are summed, so that a total maximum score of 12 indicates no impairment, while a minimum score of 0 indicates severe impairment. The first test is the limb extension, where the rat is suspended by the tail and the extension, contraction, or absence of tone is recorded. The second test is the startle response, where the rat is placed in an empty cage and their response to a loud clap sound is recorded. The third test is the beam walk, where their ability to walk across a narrow flat beam without falling or slipping limbs. The final test is the rotating beam, which measures their ability to walk on a narrow rotating suspended beam. Each rat did a baseline NAP test before receiving their first ACHI, and then repeated the procedure immediately after each injury. Cage control rats received the LOC assessment and NAP on the same timeline as the 8xACHI group.

4.2.4 Barnes Maze and Reversal

The Barnes maze and reversal tasks were used to detect impairments to spatial learning and memory, and cognitive flexibility after repeated ACHI (Barnes, 1979; Gawel, Gibula, Marszalek-Grabska, Filarowska, & Kotlinska, 2019; Rosenfeld & Ferguson, 2014; Stalnaker, Takahashi, Roesch, & Schoenbaum, 2009). Before beginning the task, the rats were allowed to acclimatize to the testing room in their home cages for 30 minutes, and to the handler for two minutes. The Barnes maze apparatus (Rat; Maze engineers, Skokie, IL) was a 122 centimetre diameter flat circular maze surface elevated 100 cm from the ground. The maze had no walls, and had 20 ten centimeter diameter holes evenly spaced around the outer edge **(Fig. 4.1)**. The maze was brightly lit, and surrounded by high contrast extra-maze visual cues. The researcher

was behind a curtain, 150 centimeters from the maze. One of the holes led to a dark box, and the rats are naturally motivated to enter the box to escape the brightly lit maze. The purpose of the task is for rodents to learn to use the distal visual cues to navigate to the escape hole over a series of training trials. In each training trial, the rat was placed in the center of the maze and allowed to explore freely for five minutes. If they found and entered the escape box in that time

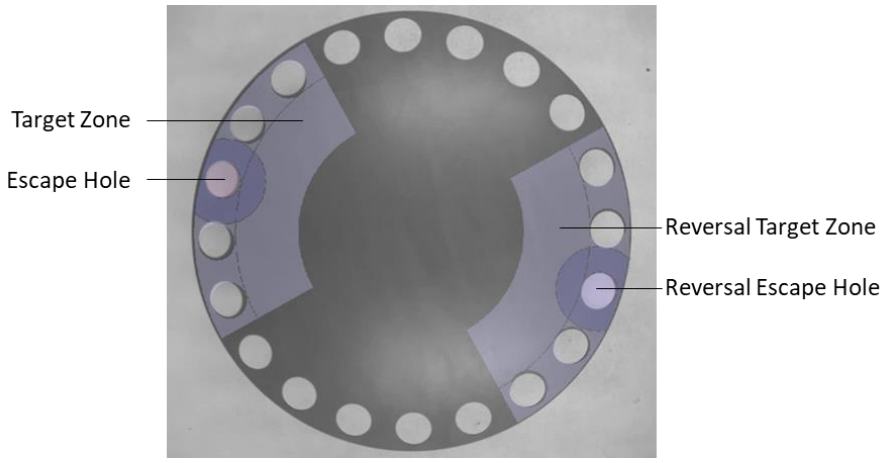


Figure 4.1: Barnes maze and reversal apparatus showing key areas of measurement for probe trials. Time in the target zone includes the total duration of time spent within the area labelled (Reversal) Target Zone, including the Escape Hole. Number of escape hole visits was calculated as the total number of times any part of the head crossed over the escape hole. Since repeat ACHI may lead to motor impairment, the total distance travelled and average speed of rats in the probe trail was measure in order to determine whether changes in motor function after ACHI affected Barnes maze performance. The maze diameter was 122cm, and it was elevated 100cm off the ground. The escape-hole diameter was 10cm.

period, they remained there for 15 seconds before being returned to their home cage. If they did not find the escape box in five minutes, the handler gently guided them there and then placed them inside where they remained for 15 seconds before being returned to the home cage. In this case rats received four trials per day for two days (total eight) with a minimum 30 minute interval between trials.

Rats were trained before receiving ACHIs, and then tested in a probe trial 1-hour after the final injury. The probe tests their ability to remember the location of the escape hole by

removing the escape box, and then allowing them to freely explore the maze for 90 seconds. The amount of time spent in the target zone and the number of times the escape hole was approached indicated how well the location was remembered.

In the reversal task, the escape box location was moved 180° to the opposite side of the maze, and the rats were re-trained to learn the new location. Beginning on PID 2 rats received three reversal training trials per day for two days (total six) with a minimum of 30 minutes between trials. One hour after the final training trial on PID 3 rats did the reversal probe test. In the reversal probe the escape box was removed, and the rats were allowed to freely explore the maze for 90s. The amount of time spent in the new target zone, and the number of times the new escape hole was approached were determined as an indicator of how well the new location was remembered. In both probe trials, velocity and distance travelled were also measured in order to rule out motor impairments. EthoVision XT 11.5 software (Noldus, Netherlands) was used to analyse tracks captured from a ceiling-mounted camera.

4.2.5 FD NeuroSilver™ II Histology

After behavioural testing, tissue was processed for histology to identify neurodegeneration on PID 3 and 7. Rats were humanely euthanized with isoflurane overdose (>5% inhalant) and immediate transcardial perfusion of 150ml PBS and 200ml 4% formaldehyde was performed. After perfusion, full brains were extracted and post-fixed in 4% formaldehyde in PBS for 48 hours, then transferred to a 30% sucrose with 0.05% sodium azide for a minimum of 48 hours, all at 4°C. The brains were submerged in ice cold PBS, and 50 μm coronal slices were taken using a Vibratome 3000 (Ted Pella Inc., Redding, CA, USA). Only slices that included a cross section of the hippocampus were used in this experiment. Slices were

distributed sequentially into six samples containing PBS with 0.05% sodium azide for storage at 4°C. Each sample included four to eight coronal slices spaced 250μm apart. One of these samples (four to eight slices) per animal was stained in this experiment.

Slices were incubated in 4% formaldehyde in saline-free phosphate buffer (0.1M) for a minimum of six days, then processed with the FD NeuroSilver™ II kit (FD NeuroTechnologies, 1997, 2018; Dhananjay R Namjoshi et al., 2014; Switzer, 2000). The kit included proprietary solutions A-G, and a standardized protocol which we optimized with minor modification as follows. They were stained in a 6-well plate with nylon well nets, and staining was carried out at room temperature with moderately vigorous mechanical agitation unless otherwise stated. Slices were rinsed with two washes of distilled water for five minutes. They were incubated in solution AB twice for ten minutes each, and then for ten minutes in solution ABE. Sections were transferred to solution CF for two minutes, twice, with vigorous manual agitation. Slices were covered from light for the remaining steps and subsequent storage. Slices were transferred to solution DF for five minutes with vigorous manual shaking. They were rinsed with distilled water twice for three minutes each, then transferred to 1X solution G for two five-minute washes. They were left in the second wash of 1x solution G for one hour at 4°C with gentle agitation.

Slices were mounted in 1X solution G onto SuperFrost slides, and allowed to dry overnight at room temperature in a fume hood. Slides were cleared with Citrisolv three times for three minutes each, and then cover-slipped using Permount. Slides were allowed to dry overnight at room temperature before imaging. Slices and ROIs that were damaged during

processing were excluded from imaging, and in the end a minimum of four slices per animal were used for further imaging and analysis.

Mounted slices were imaged with an Olympus bright field BX51TF microscope (MBF Bioscience, Williston, VT, USA). In order to account for potential between-batch staining differences, light intensity was standardized to 0.7 transmittance for each slide. StereoInvestigator software version 11.03 (MBF Bioscience, Williston, VT, USA) was used to capture selected regions of interest (ROI): the optic tract, hippocampus, corpus callosum, and cortex. A very low magnification image (4x objective) was obtained of each section, and this image was used to manually trace each region of interest ROI according to atlas boundaries (Paxinos, Watson, Pennisi, & Topple, 1985). Each ROI was then imaged completely at higher magnification (20x objective). The higher magnification images were processed in FIJI to determine the percentage of the area in each ROI that was positive for silver stain uptake. To measure this, each ROI was manually traced, and then the Savuola auto filter was applied with radius 9. This created a black and white image in which all pixels with dense enough silver uptake to meet the filters threshold were assigned a black colour value, and the remaining pixels with very weak or no silver uptake were assigned white. The percentage area stained in each ROI, or the percentage of black pixels in the ROI, was determined using these thresholded images.

How does FD NeuroSilver™ histology identify degenerating neurons?

The FD NeuroSilver™ II kit is well established in its ability to detect degenerating neurons (FD NeuroTechnologies, 2018), but FD NeuroTechnologies do not disclose the solution contents, and provide a vague summary of the mechanism by which silver granules are able to

selectively deposit on degenerating neurons. They explain that under conditions created by proprietary kit solutions, the lysosomes, axons, and terminals of degenerating neurons become argyrophilic, and readily bind to silver ions. Under further processing, silver ions form metallic granules that can be seen under an electron or light microscope (FD NeuroTechnologies, 1997).

Silver-staining to detect neural cytoarchitecture is not a novel technique. It has been over a century since the Nobel Prize for Physiology and Medicine was awarded to Camillo Golgi and Ramón y Cajal in recognition of their work elucidating neural structure using Golgi's silver-stain technique (reviewed in (E. G. Jones, 1999)). Around the same time, silver-staining was in common use in neuropathological studies, and was famously used by Alois Alzheimer to identify senile plaques (i.e. Amyloid plaques), neurofibrillary tangles, and Pick bodies in patients displaying "certain peculiar diseases of old age" decades before the biological composition of these pathological deposits were revealed ((Alzheimer, 1911); English translation (Alzheimer, Förstl, & Levy, 1991)).

Today, many variations of silver-stain remain common tools for characterisation of neural microstructure and pathology. The variations differentially process tissue to direct silver aggregation to sites of interest. For example, aggregation is facilitated by electromagnetic attraction between silver ions and sites of deposition. Silver is introduced to the solution as an ion or complex salt, which will deposit on tissue elements to which it has a high electromagnetic affinity. These salt or ion deposits cannot be visualized in this form, and the silver ion or salt must be reduced to visible metallic silver through additional chemical processing. These two criteria can be manipulated to identify different structures. The affinity of silver ions to tissue elements, and the degree that silver ions are reduced to metallic silver, change in response to

several experimental factors Uchihara, 2007). These include changes in pH, temperature, silver ion concentration, and other reagent concentrations (Gallyas, 1979). Further, silver ion deposition is a gradual processes that reaches a maximum after 15 minutes under various experimental conditions (Gallyas, 1979). Thus, differential profiles of silver aggregation can be created by manipulating these conditions over time.

The specific molecular targets of aggregation, and the reasons why these are selectively stained under different conditions, are not well understood (Uchihara, 2007). Intrinsic differences in silver ionic affinity for target sites predicate this selectivity, but it also appears to be affected by interference with other colloidal metal particles (Interpreted by (Uchihara, 2007)). That is, the presence of other metal particles in solution affect the rate at which silver ions deposit at different target sites. The rates of silver ion deposition and reduction are also affected by ultrastructural tissue characteristics like fiber density. That is, very tightly packed tissue or microstructural elements may create steric hindrance that limits access to argyrophilic sites. Conversely, loosely packed tissue or soluble molecules may not possess the structural integrity to bind and retain bulky silver deposits. Certain molecules including glutathione, creatine, and adrenaline have the endogenous ability to reduce silver deposits to metallic silver, but these require additional chemical reduction for clear visualisation (Feigin & Naoumenko, 1976; Uchihara, 2007).

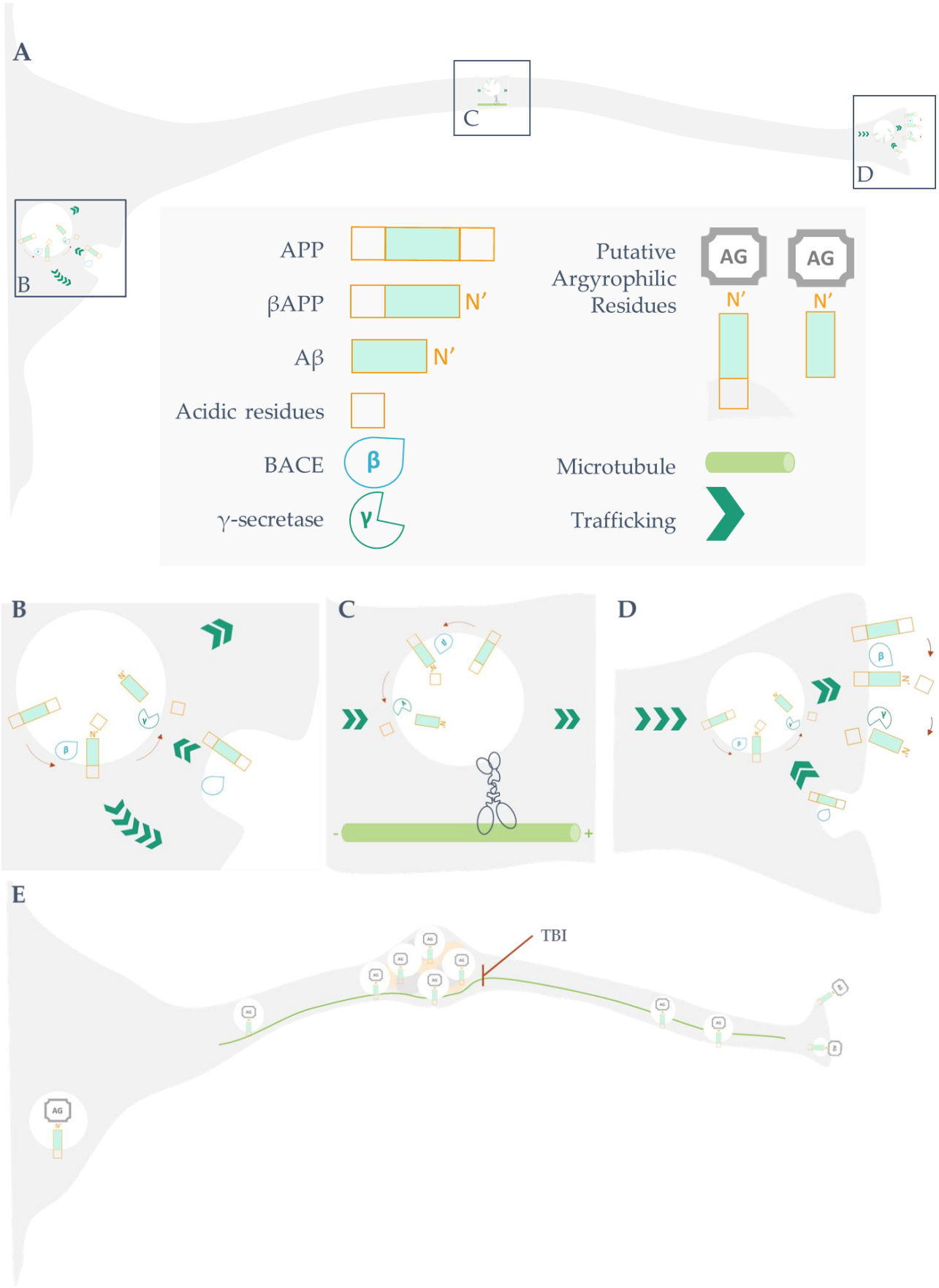
The stain characteristics observed by ourselves and others using this the FD NeuroSilver™ kit suggest it may use a variant of the Bielschowsky method, similar to a modification that was “refined by Ms. Glenna Smith” in the Yamamoto-Hirano laboratory in the late 1900s (Uchihara, 2007; Yamamoto & Hirano, 1986). This method selectively stains

degenerating axons and terminals, and has strong positivity for Amyloid plaques. It is differentiated from other common silver stain approaches in that it does not have very strong affinity for neurofibrillary tangles (Uchihara, 2007). The staining pattern we observed fits this profile, and other studies using this kit have observed positive staining in degenerating axons and Amyloid plaques (Broglio et al., 2018; W. H. Cheng et al., 2019; FD NeuroTechnologies, 2018; Liu et al., 2008; D R Namjoshi, Martin, Donkin, Wilkinson, & Stukas, 2013). Thus, the argyrophilic target(s) of FD NeuroSilver™ II histology must be abundant in degenerating axons and Amyloid plaques, but not in neurofibrillary tangles. There may be numerous molecules that fit this description, and the stain may reflect deposition at several different target sites that become more argyrophilic in degenerating neurons, but the β-amyloid (Aβ) protein and its precursors are plausible candidates.

It is possible β-amyloid precursor protein (βAPP) is a site of silver deposition that facilitates selective labelling of degenerating axons by FD NeuroSilver™ histology. The composition of Amyloid plaques is well-characterized (Atwood, Martins, Smith, & Perry, 2002). As their name suggests, Amyloid plaques are primarily Aβ polymers, but they can also associate with other proteins and metal particles. A parsimonious explanation for high FD NeuroSilver™-stain positivity in Amyloid plaques is that silver aggregates at a site found on Aβ. Aβ is well known to associate with metal ions (Tiiman et al., 2016; Wallin, Friedemann, et al., 2020; Wallin et al., 2016, 2017), and an *in vitro* study found that silver specifically aggregates at the N-terminus of the Aβ protein (Wallin, Jarvet, et al., 2020). This was discussed in the context of discovering methods of reducing the rate of Aβ polymerisation in AD, since they found silver deposition at the N-terminus of Aβ transiently reduced Aβ fibril formation. This

finding may have significant implications here as well. Silver deposition on Aβ does not explain why FD-NeuroSilver™ staining is seen in degenerating axons, since Aβ is a soluble extracellular protein. Polymerized Aβ within plaques may have sufficient structural integrity to support metallic silver deposition, but soluble Aβ likely cannot support a similar extent of deposition, or may be washed away during tissue processing. However, it is possible that an Aβ precursor retains the argyrophilic property at the N-terminus, is localized in axons, and provides a more structurally stable site for silver-deposition.

Owing to its hallmark status in Alzheimer's pathology, Aβ is well characterized. It is derived from sequential splicing of the membrane-bound amyloid precursor protein (APP) (reviewed by (J. Z. A. Tan & Gleeson, 2019)). Under normal circumstances, APP is processed into non-toxic products. It appears to have a role in synaptic neurotransmission and axonal transport, and has been shown to complex and co-transport to the presynaptic terminal with $GABA_B$ receptor 1a-containing dimers (Tang, 2019). In pathogenic circumstances, under what is termed the amyloidogenic pathway of APP processing, APP is cleaved on the extracellular membrane surface by β-secretase (BACE1), leaving membrane-bound βAPP **(Fig. 5.1 B-D)** (J. Z. A. Tan & Gleeson, 2019). βAPP is then cleaved by gamma secretase into Aβ, which dissociates from the membrane on the extracellular surface. Notably, BACE1 cleaves APP at the site that eventually becomes the N-terminus of the Aβ protein (**Fig 5.1)**. That means the extracellular exposed N-terminus of the membrane-bound βAPP is analogous to the N-terminus of Aβ. It is unclear whether structural reorganisation that would affect argyrophilic properties of the N-terminus occurs when βAPP is cleaved into Aβ. If the argyrophilic property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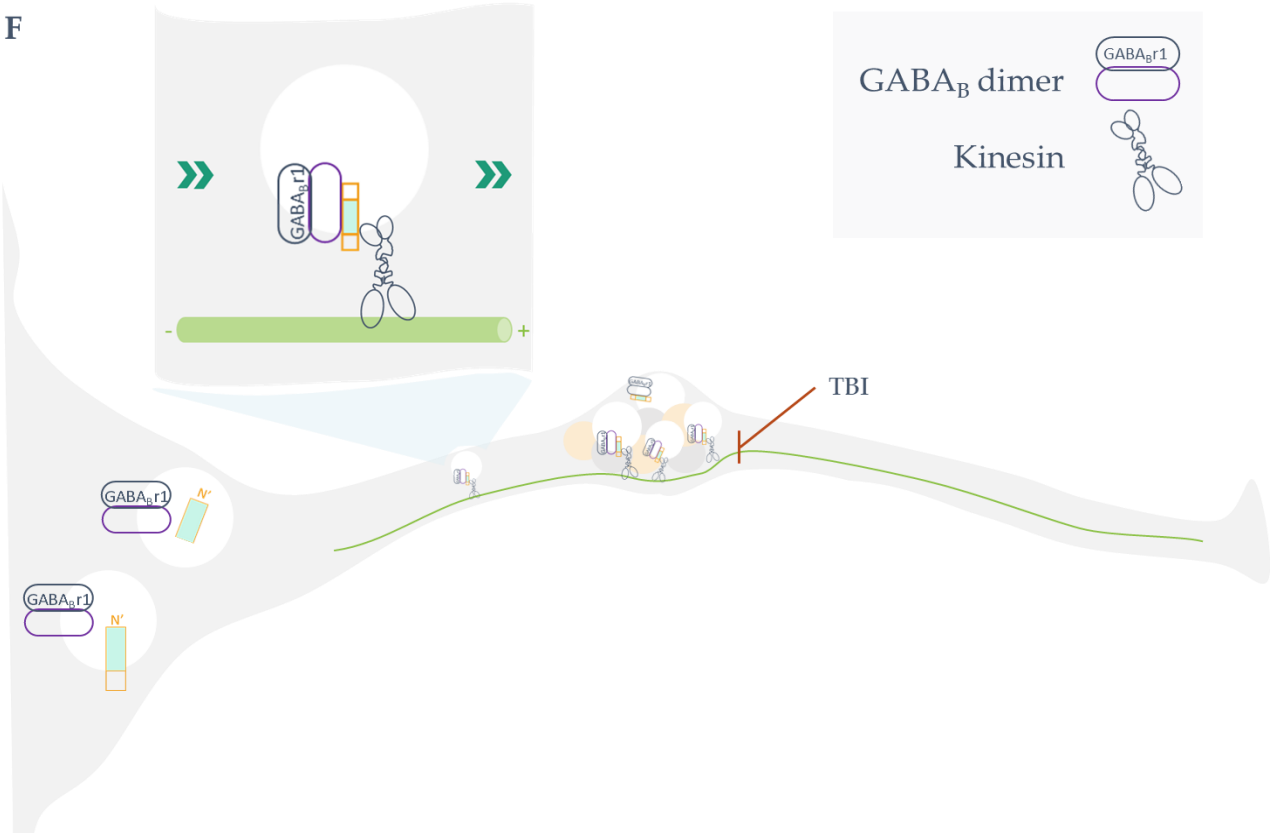


Figure 4.2: Amyloidogenic processing of APP provides a putative target of FD NeuroSilver™ stain deposition. FD NeuroSilver™ histology stains the axon, terminals, and somata of degenerating neurons, in addition to amyloid plaques. Like other silver-staining techniques, it directs the deposition of silver ions to argyrophilic target sites of interest. The experimental manipulations that facilitate specific binding is held as proprietary information, and the molecular site of silver aggregation remains unknown. Convergent evidence from the literature suggests the N-terminus of βAPP is a putative target of silver aggregation in degenerating neurons. **(A)** βAPP secretion has been demonstrated in the somata, axons, and terminals of neurons. **(B-D)** The amyloidogenic pathway of APP processing involves sequential cleavage of membrane-bound APP to βAPP, and then soluble Aβ. This proteolytic cleavage is mediated by BACE1 and γ-secretase, respectively. APP processing shifts towards the amyloidogenic pathway in pathologic conditions including TBI. Amyloidogenic APP processing is facilitated by endocytic sequestration of APP and BACE1. There are several trafficking steps where APP and BACE1 can co-localize in vesicles, including **(B)** somatic endosomes/lysosomes, **(C)** axonal transport vesicles, and **(D)** at presynaptic terminals. Amyloidogenic processing can also occur on the cell membrane, especially at the presynaptic terminal. **(E)** After FD NeuroSilver™ histology, any APP that has been cleaved by BACE1, but not yet by γ-secratase, remains membrane bound but has an exposed, putatively argyrophilic N-terminus. This can occur anywhere APP and BACE1 are sequestered together, i.e. the somatic exosomes/lysosomes, axons, and terminals of degenerating neurons. These are also the sites detected by FD NeuroSilver histology, thus βAPP is a putative target of this staining technique. **(F)** With respect to disruption of GABAergic signalling after TBI: APP can form complexes with GABA$_B$ receptor dimers containing at least one R1 subunit, which appears to have a role in anterograde transport of GABA$_B$r1. Amyloidogenic processing of APP might prevent complexing with GABAb dimers, thus preventing the initiation of anterograde transport. Alternatively it is possible amyloidogeneic processing does not significantly affect complexing or transport initiation of GABA$_B$ dimers, but they do not reach the terminal because all axonal transport is affected by the energetic crisis and cytoskeletal disruption resulting from TBI. (Aβ amyloid beta; AG silver; APP amyloid precursor protein; βAPP beta amyloid precursor protein; BACE β-secretase; GABA gamma-aminobutyric acid; GABA$_B$ GABA B type receptor; GABA$_B$r1 GABA B type receptor with R1 subunit)

is retained, βAPP represents a neuronal membrane-bound argyrophilic site that is more abundant in pathogenic circumstances. Furthermore, amyloidogenic APP processing can occur wherever APP and BACE1 trafficking converge, including in lysosomes and endosomes in the soma (Pasternak, Callahan, & Mahuran, 2004), axonal transport vesicles, and on membranes and endosomes at the presynaptic terminal. (J. Z. A. Tan & Gleeson, 2019). Importantly, βAPP secretion has been observed in axons, somata, and terminals (Niederst, Reyna, & Goldstein, 2015) (**Fig 5.1)**. Thus, the staining profile that would be expected from silver aggregation at the N-terminals of Aβ and βAPP is identical to what is seen in FD NeuroSilver™ histology.

The amyloidogenic pathway of APP processing is not exclusive to Alzheimer's disease, and Amyloid plaques are seen to a lesser extent than Alzheimer's in multiple neurodegenerative disorders. This notably includes CTE, a progressive neurodegenerative tauopathy caused by repetitive mild traumatic brain injury (Martland, 1928; McKee et al., 2009; Mez et al., 2017). There is considerable evidence TBI promotes amyloidogenesis (Blasko et al., 2004; Uryu et al., 2007) (reviewed in (Edwards, Moreno-Gonzalez, & Soto, 2017)). A shift in APP processing towards the amyloidogenic pathway requires an increase in BACE1 activity. Precisely how this occurs after concussion is still under investigation, but it appears that BACE1 transcription factors are regulated by neuroinflammatory signalling pathways. For example, BACE1 expression is upregulated by NF-κB (C. H. Chen et al., 2012), a transcription factor with a complex role in neuroinflammation, which is upregulated after TBI (Merchant-Borna et al., 2016; Sanz, Acarin, González, & Castellano, 2002). Importantly, immunohistochemical staining has confirmed axonal localization of BACE1 (Uryu et al., 2007) and βAPP after TBI (Gentleman et al., 1993; Sblano et al., 2012; Sherriff, Bridges, & Sivaloganathan, 1994).

The co-localization of APP and $GABA_A$ in transport vesicles is interesting in light of a recent finding that GABAergic signalling is disrupted after TBI, and this is accompanied by a reduction in $GABA_A$ and $GABA_B$ receptors at the terminal (Parga Becerra, Logsdon, Banks, & Ransom, 2021). If APP has a functional role in GABA receptor transport to the synapse, it is possible that an injury-induced transition to the amyloidogenic pathway disrupts GABA receptor transport. The disruption of bidirectional axonal transport is an established concussion mechanism, but it is thought primarily to be driven by cytoskeletal and microtubule damage, and energetic crisis (Christopher C Giza & Hovda, 2014). Thus, amyloidogenic APP processing may be coincidental rather than causal to GABAergic signalling disruption.

Axonal varicosities are often visible in traumatized neurons stained with FD NeuroSilver™ . This suggests that silver deposition may occur on a molecule involved in axonal transport, which accumulates in axonal varicosities due to TBI-induced transport disruptions (Tang-Schomer et al., 2012). The widened portions of varicosities may represent sites where transport vesicles and apparatus accumulate due to transport disruptions and microtubule damage (Tang-Schomer et al., 2012). Axonal varicosities are also present in normal unmyelinated axons where they are often found at presynaptic boutons, and the membrane diameter differentials are thought to modulate electrical signalling (Shepherd, Raastad, & Andersen, 2002). TBI leads to increased formation of axonal varicosities (Tang-Schomer et al., 2012), which may represent a new form of mechanosensitive modulation of plasticity (Gu, 2021).

All together this suggests the N-terminus of βAPP may be a target of silver aggregation in FD NeuroSilver™-staining. If this is the case, it also suggests that amyloidogenic processing

of APP is an outcome of ACHI. Future experiments with the ACHI model should explore the extent to which injury promotes amyloidogenesis, for example through immunohistochemistry, or molecular assays for changes in the distribution and relative abundance of BACE1 or βAPP and other APP variants. FD NeuroSilver™ can also be visualized using electron microscopy, which may be used to explore the ultrastructural localisation of silver deposition.

4.2.6 Statistics and Graphing

Data were organized on Microsoft Excel 2016 (Microsoft, Redmond, WA), and analyzed in R Studio version 3.6.2 (RStudio, PBC, Boston, MA). Graphs were created in Microsoft Excel or R studio. NAP scores across all trials were analysed using mixed factorial ANOVA with injury group and sex as between-subjects factors and ACHI number as within-subjects factors. Interactions were assessed using *post hoc* pairwise comparisons. A Greenhouse Geisser correction was used to correct for departure from sphericity. The NAP score after the final ACHI was compared to control using a Wilcoxon rank-sum test with a continuity correction. Barnes maze and reversal training data were analyzed using mixed factorial ANOVA with trial number as within-subjects factor, and sex and injury group as between-subjects factors. Interactions were investigated using pairwise comparisons. The probe trials were analyzed using factorial ANOVA with sex and injury as between-subjects factors.

FD NeuroSilver™ histological data were assessed using factorial ANOVA with injury group and sex as between-subjects factors. This was done for each day (i.e. PID 3 and 7) and for each ROI (i.e. optic tract, hippocampus, corpus callosum, and cortex). The percent area stained was first calculated individually for each hemisphere in the hippocampus and optic tract. In these regions, the average area stained were compared between hemispheres using mixed

factorial ANOVA with sex and injury as between-subject factors, and hemisphere as within-subject factor. There were no significant effects or interactions of hemisphere, so the results for each hemisphere were pooled for the analysis and findings described in Figure 3B.

An independent sample two tailed T-Test was used to compare weight between controls and 8xACHI rats at the time of their experimental endpoints. Welches adaptation of the student T-test was used to account for unequal variances between groups.

4.3 Results

4.3.1 Subjects

A total of 32 juvenile Long Evans rats were used with an equal number of males and females. Half of these rats received eight ACHIs beginning on postnatal day (PND) 25-26, and the rest were age- and sex- matched cage controls. All original rats met their experimental endpoints, which were three and seven days after the final ACHI. The experimental timelines are summarized in **Figure 4.2A**, and group sizes and breakdowns are shown in **Figure 4.2**B. Weights were measured at experimental endpoints, and there were no differences in the average weight of control or 8xACHI groups, regardless of sex. On PID 3, the average weight of female cage controls was 93.75 g, which was not significantly different than the female 8x ACHI group at 95g, as confirmed by Welches two-sample t-test [T_6=0.1412, p=0.8924]. Similarly, cage control males weighed an average of 97.75g, and 8xACHI males weighed an average of 956, which was not significantly different [$T_{(5.836)}$ =0.2928, p=0.7798]. On PID 7 the cage control females weighed an average of 144.5g, which was not significantly different than those that

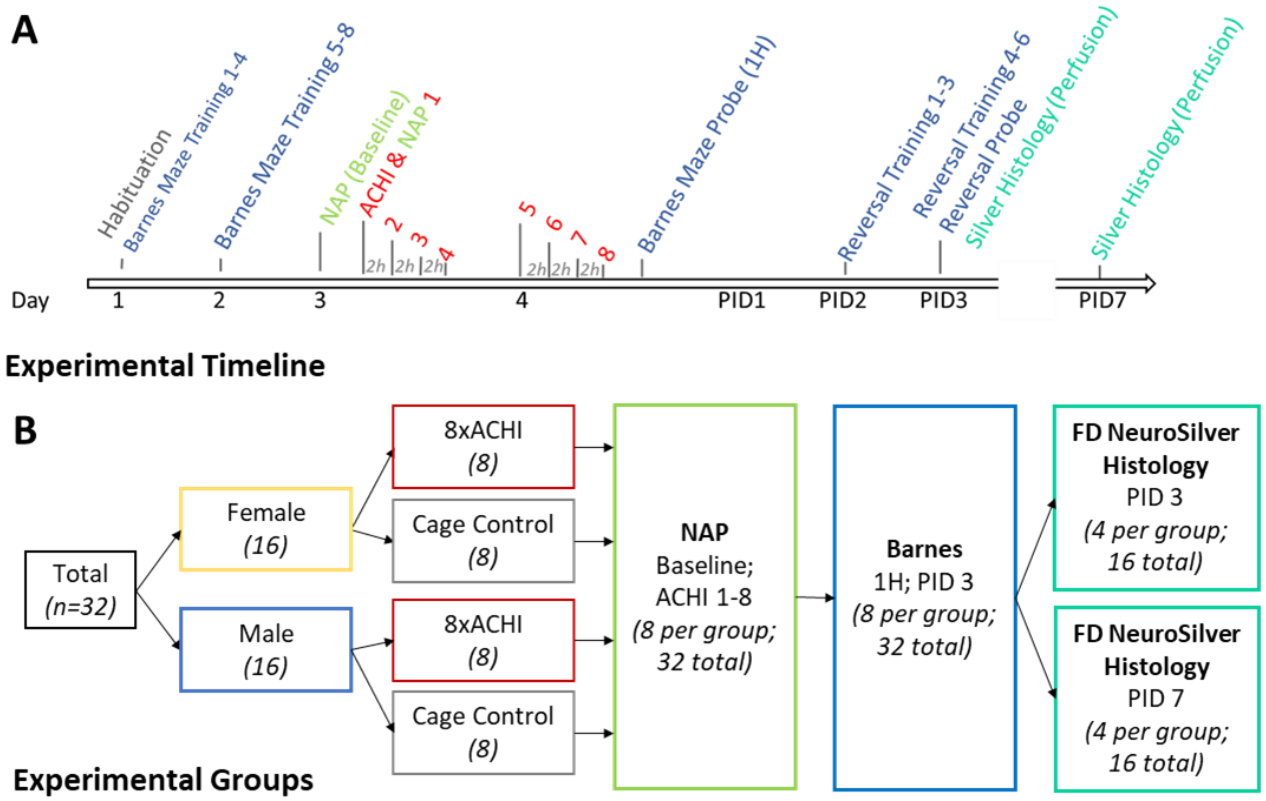


Figure 4.3: Experimental timeline and group breakdown for behavioural and histological characterisation of repeated ACHI. (A) On the first day rats were habituated to the handler for two minutes, and to the testing room for thirty. The following two days included four trials each of Barnes maze acquisition training. On day three, all rats completed the baseline NAP, then were pseudo-randomized into the control or 8xACHI group. Littermates and cage-mates were distributed evenly between groups. The 8xACHI group received four head impacts per day, for 2 days, with 2 hours between each injury (total 8 injuries) using the ACHI model. They were assessed using the NAP immediately after each ACHI. The control group did the NAP tests on the same timeline as the 8xACHI group. The Barnes maze probe test occurred one hour after the final ACHI, on day four. Barnes reversal training happened on PID 2-3, which included three trials per day. Rats did the reversal probe trial one hour after the final reversal training trial. One hour later, half of the rats were killed via transcardial perfusion, so that tissue could be extracted for FD NeuroSilver™ histology. The other half were perfused on PID 7. **(B)** A total of 32 juvenile Long Evans rats were used for these experiments. The first ACHI or NAP was completed on postnatal day 25-26 (PND 25-26). The 32 rats included an even number of females and males (16 each). They were split evenly into 8xACHI or cage control groups. The 8xACHI group received eight ACHIs over two days, while the cage control remained in their cages. Both groups underwent all NAP and Barnes maze training and testing, such that there were a total of eight animals in each of the four injury groups (i.e. control female; 8xACHI female; control male; 8x ACHI male). After NAP and Barnes testing was complete, these groups were split evenly so that four per group were perfused for FD NeuroSilver™ stain histology on PID3, and the other four on PID7. (ACHI awake closed head injury; NAP neurologic assessment protocol; PID post-injury day; PND postnatal day)

received 8xACHI at 137.75g [$T_{(5.981)}$=0.3750, p=0.7206]. The average weight of the male cage controls was 123.25g, which was not significantly different than males that received 8xACHI at 116g [$T_{(5.512)}$=0.7792, p=0.468].

3.2 Loss of Consciousness

All rats were assessed for LOC at baseline, and immediately after each injury. No rats at baseline, and no control rats showed signs of changes in consciousness. No apnea was observed. A small subset of rats in the 8xACHI group had impaired toe pinch and righting reflexes. The smaller subset of data was statistically underpowered, but can be summarized as follows. There was a five second delay in toe pinch reflex in one female after the sixth injury, and in one male after the eighth injury. **Figure 4.3** shows the latency to self righting after each ACHI. Six females and seven males experienced at least one instance of delayed righting reflex. In total, 33 out of the 272, or (12.5%) of impacts resulted in a delayed righting reflex. Within this subset of rats that had a delayed righting reflex, the average latency to self right was six seconds in the females and 31 seconds in males.

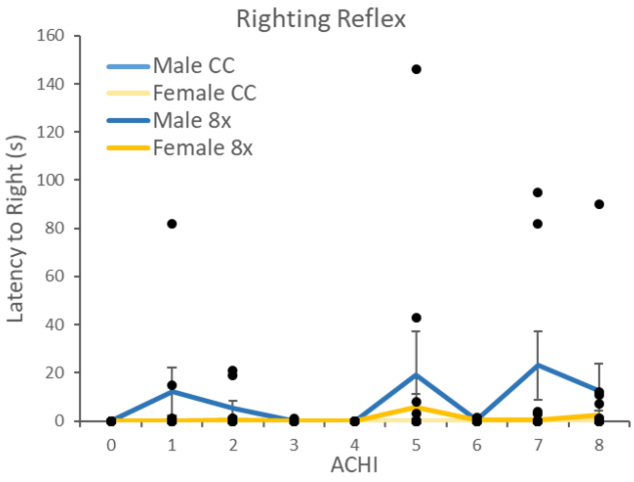


Figure 4.4: Righting reflex was impaired in a subset of rats. Rats were placed on their back, and the latency to flip into an upright position was measured. All rats in the control group self-righted immediately in every trial. Six females and seven males in the 8xACHI group experienced at least one instance of delayed righting reflex. Within this subset of rats, there were 14 instances of delayed righting reflex in the females with an average latency to self right of six seconds, and there were 19 instances of delayed righting reflex in the males with an average latency to self right of 31 seconds. Due to the data being non-parametric, with only small subset providing non-zero values, these results were not sufficiently powered for meaningful statistical analysis. (ACHI awake closed head injury)

4.3.3 Neurologic Assessment Protocol

NAP (Christie et al., 2019; Meconi et al., 2018) was administered at baseline, and immediately after each ACHI. The cage control group did NAP testing on the same timeline. A score of 12 indicates no neurologic impairment and a score of 0 indicates severe impairment. The average NAP score for each group after each ACHI is shown in **Figure 4.4A**. Rats from the 8xACHI group scored equivalently to cage controls at baseline, but their NAP score was significantly lower after the first injury and declined until the final injury.

Mixed factorial ANOVA confirmed significant main effects of injury [$F_{(1, 28)}$=179.8, p<0.0001, ges=0.63], of sex [$F_{(1, 28)}$ =5.38, p=0.03, ges=0.05], and of ACHI number [$F_{(4.02, 112.52)}$

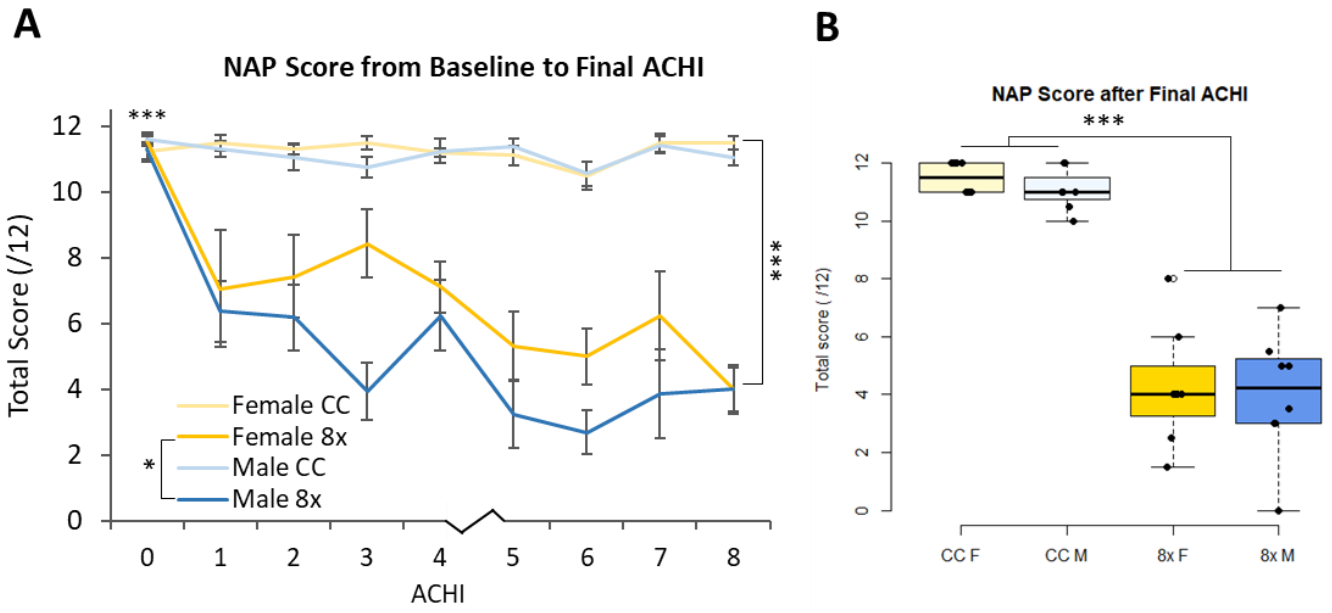


Figure 4.5: NAP score was significantly impaired after 8xACHI. Neurologic function was measured using the NAP at baseline before the first injury, and immediately after each injury. The NAP consists of 4 simple tests of basic motor and reflex function, each scored from 0-3. The total NAP score is the sum of the scores for each task, with a maximum score of 12 indicating no impairment and a minimum score of 0 indicating the most severe impairment. **(A)** 8x ACHI significantly impaired NAP performance after a single impact, and impairment persisted through the final impact. At baseline, all groups performed equivalently and achieved a near-maximum score. In the 8xACHI group only, males performed worse than females (*). **(B)** After the final injury the 8xACHI group performed significantly worse than the control group. (*: p<0.05; **: p<0.01; ***: all p<0.001| ACHI awake closed head injury; NAP neurologic assessment protocol; PID post injury day)

=13.76, p<0.001, ges=0.26]. There were significant interactions between injury*ACHI [$F_{(4.02, 112.52)}$ =11.32, p<0.001, ges=0.23] and injury*sex [$F_{(1, 28)}$ = 4.33, p=.046, ges=0.04]. A Greenhouse Geisser correction was used to correct for departure from sphericity in calculating main effects and interactions of ACHI number.

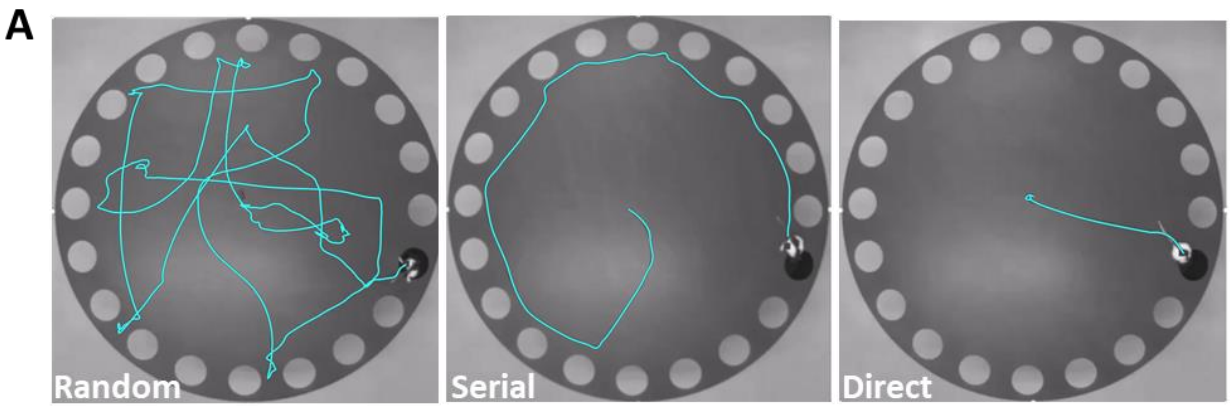
Post hoc pairwise contrasts to examine the main effect of ACHI number showed that baseline performance was better than all other tests (all p<0.0001). Pairwise contrasts to examine the injury group*ACHI number interaction found the control group performed equivalently to the 8xACHI group at baseline, with scores of 11.38 and 11.44, respectively [$T_{(202)}$ =0.085, p=0.9320], and then scored significantly higher than ACHI rats in all post-injury tests (all p<0.0001). Pairwise contrasts to examine the ACHI number*injury group interaction found that 8xACHI rats performed significantly better at baseline than all other trials (all p<0.0001). Their scores in trials 1, 2, and 4 were significantly higher than in 5, 6, and 8 (all p<0.01). Pairwise comparisons to examine the sex*injury group interaction males performed worse than females in the 8xACHI group [$T_{(28)}$ = 3.11, p=0.0043]. In the control group, the sexes performed equivalently [$T_{(28)}$ = 0.169, p=0.8674].

The final NAP score, taken immediately after the 8th ACHI, is shown in **Figure 4.4B**. The 8xACHI group performed significantly worse than the control group [W=265, p<0.0001] with average scores of 4.13 and 11.28 respectively. There was no difference in performance between sexes [W=141, p=0.63] in this trial.

4.3.4 Barnes Maze

Acquisition

The Barnes Maze was used to assess spatial memory after 8xACHI. Initial habituation and training were completed before the injuries. The probe test, reversal training, and reversal probe were completed after the injuries. In the training trails rats learned to use distal visual cues to locate an escape hole in the perimeter of a circular Barnes maze. The distance traveled in each trial was measured, with a shorter distance indicating a better performance in the trial. Representative paths from this cohort show examples of three common search strategies in the Barnes maze **(Fig. 4.5A)**. Typically in early learning trials rodents are more likely to use a random search strategy, then progress to serial, then direct to find the escape (Harrison, Reiserer, Tomarken, & McDonald, 2006; Rosenfeld & Ferguson, 2014). **Figure** 4.**5B** shows the average distance travelled by each group during pre-injury training trials. We found that all four groups were able to learn the location of the escape platform equivalently by the end of the training trials. A significant main effect of trial [$F_{(3.31, 92.76)}$ =14.57, p<0.001, ges=0.32] was confirmed by mixed factorial ANOVA. Pairwise comparisons to examine the trial effect revealed that all rats travelled a significantly longer distance in trial 1 than all others (all p<0.05), and travelled longer in trial 2 and 5 than in 4, 6, 7, and 8 (all p<0.05). Importantly, all rats performed equivalently in the final three trials, during which most rats travelled directly to the escape hole after release. Unexpectedly, there was a significant interaction of sex*injury group [$F_{(1, 28)}$ =5.27, p=0.03, ges=0.02]. Pairwise comparisons to explore this interaction found that within the group that would receive 8xACHI, the males performed worse than the females (p<0.01), and *ad hoc* pairwise comparisons of the interaction between sex, injury group, and trial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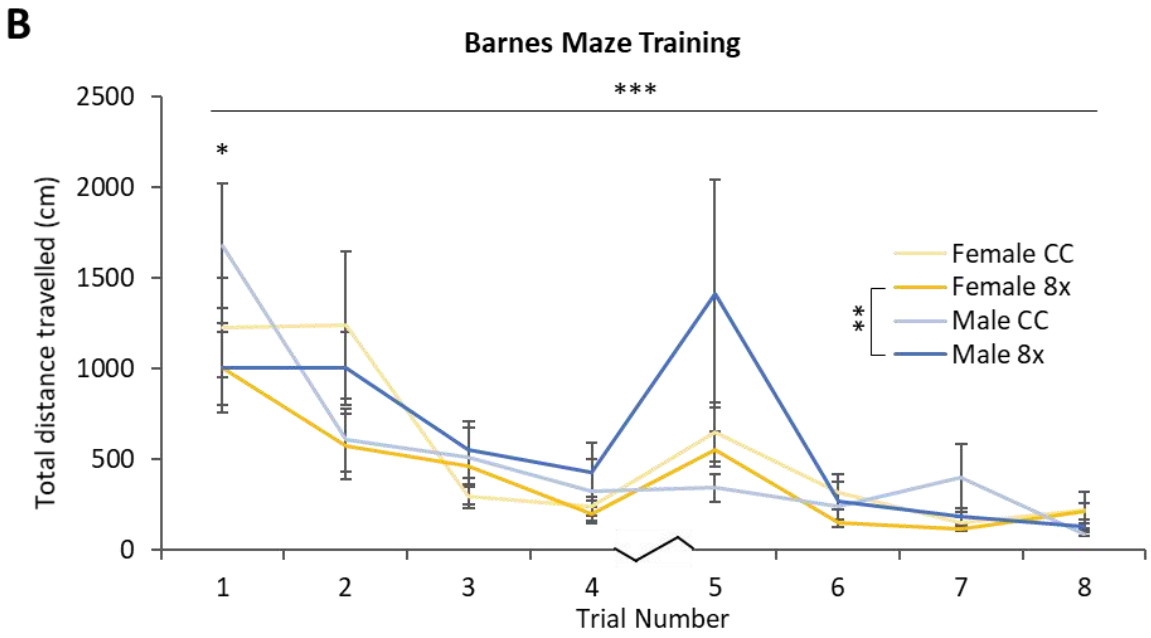
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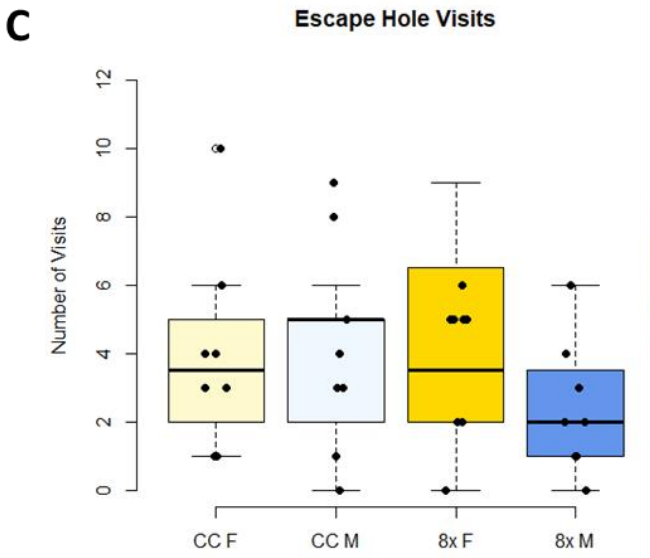
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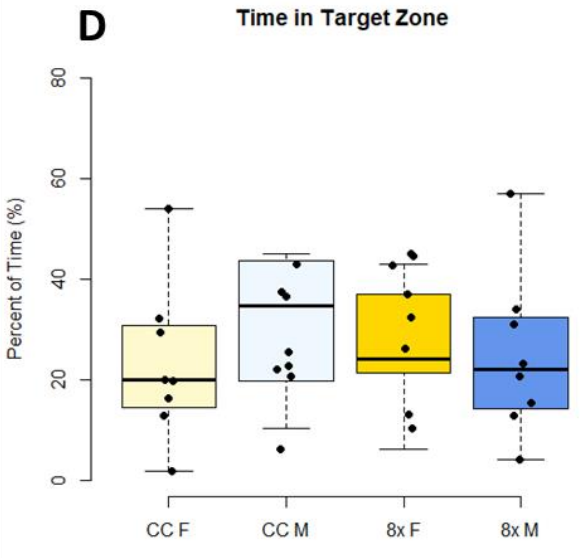
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8x F
8x M

Figure 4.6: Barnes maze acquisition probe was not affected by 8xACHI. All groups learned to locate the Barnes maze escape hole in pre-injury training, but 8xACHI did not affect memory in the probe test 1-hour after final injury. The Barnes maze was used to test spatial memory after 8xACHI. All groups were trained to use extra-maze visual cues to locate an escape hole in a brightly lit, elevated circular platform. The distance travelled by each rat in each trial was measured, and a progressively shorter path length meant they were learning the escape-hole location. **(A)** Representative paths from this cohort show examples of three common search strategies in the Barnes maze. In early learning trials rodents are more likely to use a random search strategy, then progress to serial, then direct to find the escape. **(B)** In the pre-injury training trials, all groups learned the escape-hole location by the final trial, and there were no differences in performance between groups in the final three trials. There was a significant main effect of trial (***). There was a significant interaction of injury group*sex, where males performed significantly worse than females in the 8xACHI group (**). At the time of training, all four groups had been treated exactly the same, and group differences were not hypothesized. Importantly, all groups learned the task equivalently by the final three trials. Note the break in the x axis denotes the overnight break between training trials 4 and 5. **(C-D)** In the probe test, the escape box was removed so that the rats could not enter the escape hole, and they were allowed to explore for 90 seconds. The amount of time they spent searching the target zone as well as the number of visits to the escape hole reflect the extent to which the subject remembers the task. There were no differences in performance in the probe trial. **(C)** All group visited the escape hole an equivalent number of times, and **(D)** spent an equivalent amount of time in the target zone. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$| ACHI awake closed head injury; NAP neurologic assessment protocol; PID post injury day)

showed that 8xACHI males performed significantly worse than 8xACHI females in trial five ($p<0.05$), only. This suggests the sex-difference is due to anomalously high values in the male 8xACHI group during the 5th trial. The probe trial was completed one hour after the final ACHI. The escape hole was removed and each rat was allowed to explore the maze for 90 seconds. The number of visits to the escape-hole, as well as time spent exploring the target area surrounding it were recorded. Factorial ANOVA confirmed there were no differences in performance in the probe trial. As is shown in **Figure 4.5C**, all groups visited the escape hole an equivalent number of times with no main effect of injury [$F_{(1,28)}=0.32$, $p=0.56$]; of sex [$F_{(1,28)}=0.19$, $p=0.66$], and no interaction [$F_{(1,28)}=0.68$, $p=0.42$]. Similarly, **Figure 4.5D** shows all groups spent an equivalent amount of time in the target zone with no main effect of injury [$F_{(1, 28)} = 0.71$, $p=0.41$], of sex [$F_{(1, 28)} =1.2$, $p=0.27$], and no interaction [$F_{(1, 28)} =0.99$, $p=0.33$].

Reversal

Reversal training happened on PID 2-3. The escape-hole location was moved 180°, and each rat was given six reversal training trials to learn the new location. All groups travelled further in the first trial than the final five trials, and the 8xACHI group travelled father than controls in the first trial **(Fig. 4.6A)**. Mixed factorial ANOVA confirmed a significant main effect of trial [$F_{(1.43, 39.93)} = 12.27$, $p<0.001$, ges=0.25]. Pairwise comparisons examining the trial effect showed the distance travelled in trial 1 was significantly greater than all others (all $p<0.0001$). There was a significant interaction effect of injury*trial [$F_{(1.43, 39.93)} = 4.93$, $p=0.02$, ges=0.12]. Pairwise comparisons of this interaction confirmed the 8xACHI group travelled significantly farther before finding the escape hole than controls in the first trial ($p<0.0001$), but performed equivalently in the remaining trials (all $p>0.05$).

The reversal probe trial was completed after reversal training on PID 3. The escape hole was removed and each rat was allowed to explore the maze for 90 seconds. The time spent exploring the reversal target zone and the initial target zone, as well as the number of visits to the reversal escape hole and the initial escape hole were recorded. There were no differences in performance in the reversal probe trial, which was confirmed by factorial ANOVA. All groups visited the reversal escape hole an equivalent number of times **(Fig.4.6B)** with no main effect of injury [$F_{(1, 28)} =0.02$, $p=0.88$]; of sex [$F_{(1, 28)} =0.01$, $p=0.94$]; and no interaction [$F_{(1, 28)} =0.09$, $p=0.77$]. Similarly, **Figure 4.6C** shows all groups spent an equivalent amount of time in the target zone with no main effect of injury [$F_{(1, 28)} =0.41$, $p=0.53$], or sex [$F_{(1, 28)} =0.67$, $p=0.42$], and no interaction effect [$F_{(1, 28)} =1.66$, $p=0.21$].

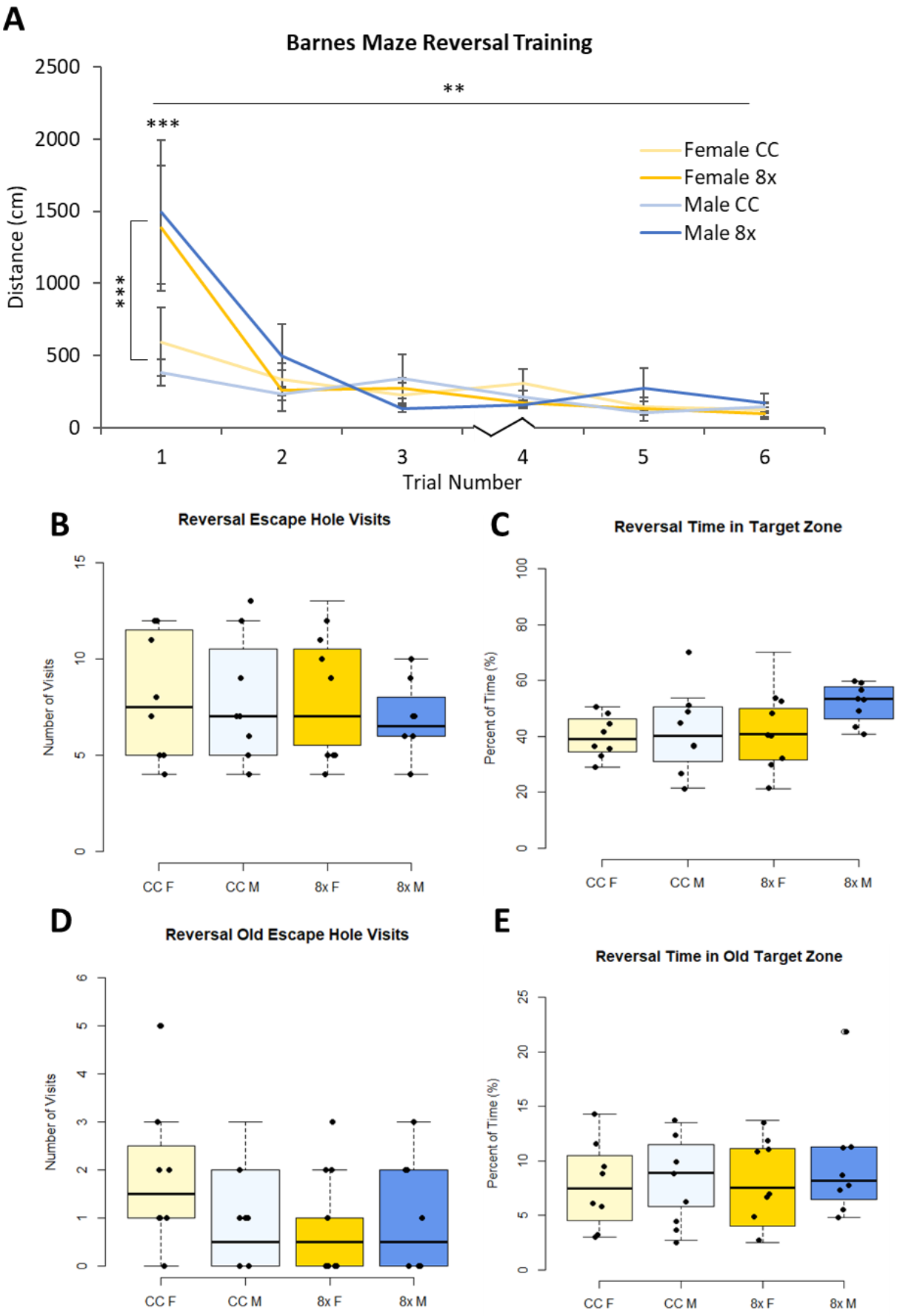
A
Barnes Maze Reversal Training
Distance (cm)
Trial Number
Female CC
Female 8x
Male CC
Male 8x
**
***
***
B
Reversal Escape Hole Visits
Number of Visits
C
Reversal Time in Target Zone
Percent of Time (%)
D
Reversal Old Escape Hole Visits
Number of Visits
E
Reversal Time in Old Target Zone
Percent of Time (%)
CC F
CC M
8x F
8x M

Figure 4.7: Barnes maze reversal learning was impaired by 8xACHI (A) On PID 2-3 rats completed reversal training. For the reversal task, the location of the escape hole was moved 180° from the initial training trials, and the rats were given 3 trials per day to learn the new location. A lower path length indicates better performance. Importantly, there is a significant interaction of injury*trial, and the 8xACHI group travelled a significantly longer distance than controls in the first trial (***). They performed equivalently to controls in the remaining trials. There was a significant main effect of trial (**). The distance travelled in trial 1 was greater than all others (***), and there were no significant differences in the distance travelled in the last three trials (all p>0.05), regardless of group or sex. This indicates that all groups learned to locate the reversal escape hole by the final trial. **(B)** In the reversal probe, the escape box was removed and the rats were allowed to explore the maze for 90 seconds. The number of visits to the reversal and initial escape holes, as well as the amount of time spent in the reversal target and old target zones were recorded. More visits to the escape hole and more time spent in the target zone indicate better memory of the task. All groups visited the escape hole an equivalent number of times, and **(C)** spent an equivalent amount of time in the target zone. There were also no significant differences in **(D)** the number of times they visited the old escape hole, or **(E)** in the time spent in the old target quadrant. (** p<0.01; *** p<0.001| ACHI awake closed head injury; PID post injury day)

During the reversal probe there were also no significant differences in the number of visits **(Fig. 4.6D)**, or amount of time spent near the initial escape hole **(Fig. 4.6E)**, which was confirmed using factorial ANOV. There was no main effect of injury [$F_{(1, 28)}$ =3.44, p=0.07], of sex [$F_{(1, 28)}$ =2.48, p=0.12], and no interaction effect [$F_{(1, 28)}$ =2.14, p=0.15] on the number of visits to the old escape hole. There was also no main effect of injury [$F_{(1, 28)}$ =0.08, p=0.77], of sex [$F_{(1, 28)}$ =0.01, p=0.91], and no interaction effect [$F_{(1, 28)}$ =0.18, p=0.67] on the time spent in the old target zone. In order to determine if NAP score correlated with Barnes maze deficits following repeated ACHI, outcomes from the acquisition probe (**Fig 4.7A**), the first reversal training trail (i.e. when deficits were observed) (**Fig. 4.7B),** and the reversal probe trial (**Fig 4.7C**) were plotted individually against NAP scores. This also allowed us to determine whether NAP scores may predict cognitive outcomes. In this case, NAP score did not predict performance in any Barnes maze metric (all $R^2 < 0.20$). Average velocity and distance travelled in the Barnes maze were measured in order to determine whether 8xACHI affected motility, (**Fig. 4.8).** No differences

were observed in the acquisition or reversal probe, indicating differences in motor function did not affect Barnes maze performance.

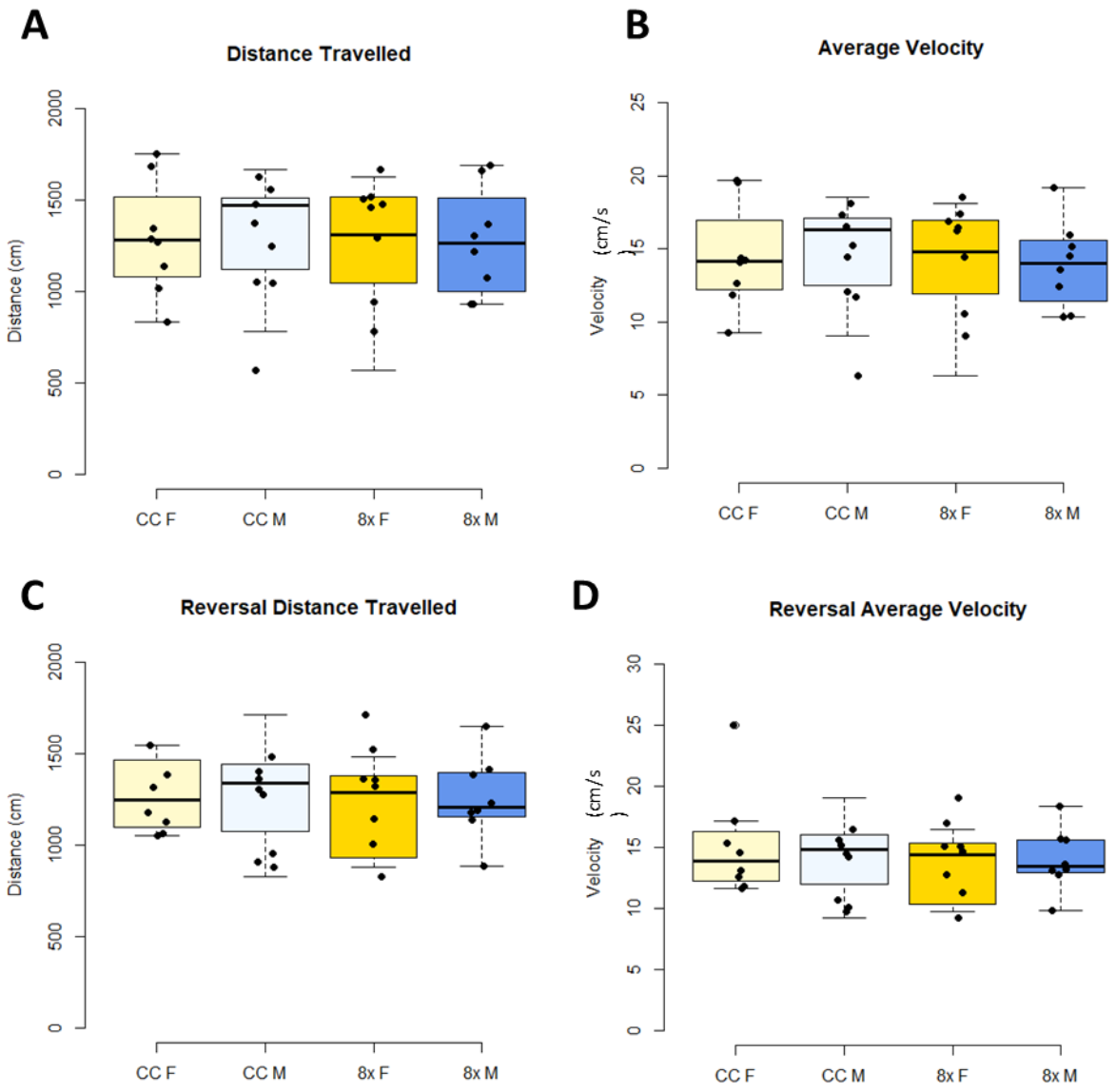

Figure 4.8: Motility in the Barnes maze was not affected by 8xACHI. Factorial ANOVA with sex and injury group as between subjects factors confirmed that all groups travelled an equivalent distance **(A)** at an equivalent velocity **(B)** in the probe trial. There were also no significant differences in distance travelled **(C)** or average velocity **(D)** in the reversal probe. (ACHI awake closed head injury; CC cage control; F female; M male)

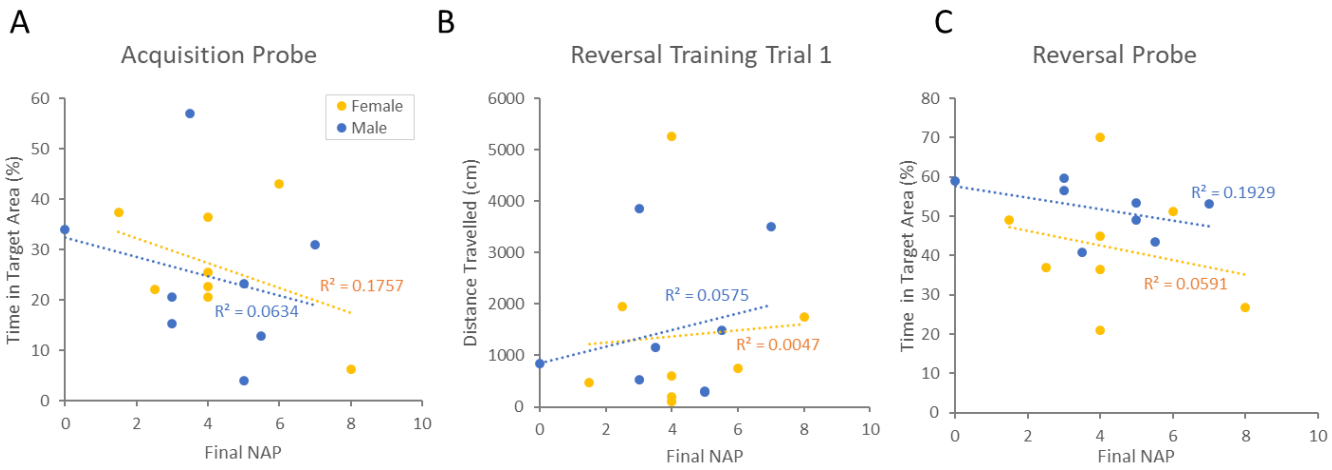

Figure 4.9: NAP score did not predict performance in the Barns maze or reversal after 8xACHI. In the 8xACHI group only, NAP score was compared to **(A)** the amount of time in the target zone during the Barnes maze probe, **(B)** the latecy to escape in the first Barnes reversal trial where deficits were found and **(C)** to time time spent in target zone during the reversal probe. Linear trendlines for each sex are shown. No notable correlations were observed (all $R^2 < 0.2$).(ACHI awake closed head injury; NAP neurologic assessment protocol)

4.3.5 FD NeuroSilver™ Histology

FD NeuroSilver™ II (FD Technologies, Columbia, MD) histology was completed on the third and seventh day after 8xACHI to identify neurodegeneration in four regions of interest: the bilateral hippocampus and optic tract; the corpus callosum, and the ipsilateral injury cortex. **Figure 4.9A** shows representative coronal cross sections of silver stained tissue, and **4.9B** shows representative images from the optic tract selected *ad hoc* to show an example of silver stain deposition density and patterns. In the optic tract, linear aggregation of silver granules in spaces not overlapping with cellular nuclei in white matter tracts suggest stain uptake might be associated with axons. **Figure 4.10** shows additional representative cross sections and magnifications that demonstrate silver stain depositions patterns in each ROI. In the representative cross sections and particularly in the optic tract magnifications, positively stained tissue with uptake of grey and black granules is more prominently visible in the 8xACHI examples than control. The percentage of the area of each ROI that is positively stained was compared between groups **(Fig. 4.9C).** Factorial ANOVA confirmed that on PID 3 the 8xACHI group had a significantly greater stained area in compared to control in the optic tract [$F_{(1,28)}$=17.62, $p<0.001$] and hippocampus [$F_{(1,28)}$=5.51, $p=0.026$]. On PID 7 the 8xACHI group also had a significantly greater area stained compared to control in the optic tract [$F_{(1, 28)}$ =20.39, $p<0.001$], hippocampus [$F_{(1, 28)}$ =9.34, $p=0.005$], and cortex [$F_{(1, 12)}$ =6.39, $p=0.026$ on PID 7]. There was a significant main effect of sex in the hippocampus on PID 7 [$F_{(1, 28)}$ =4.9884, $p=0.033$]

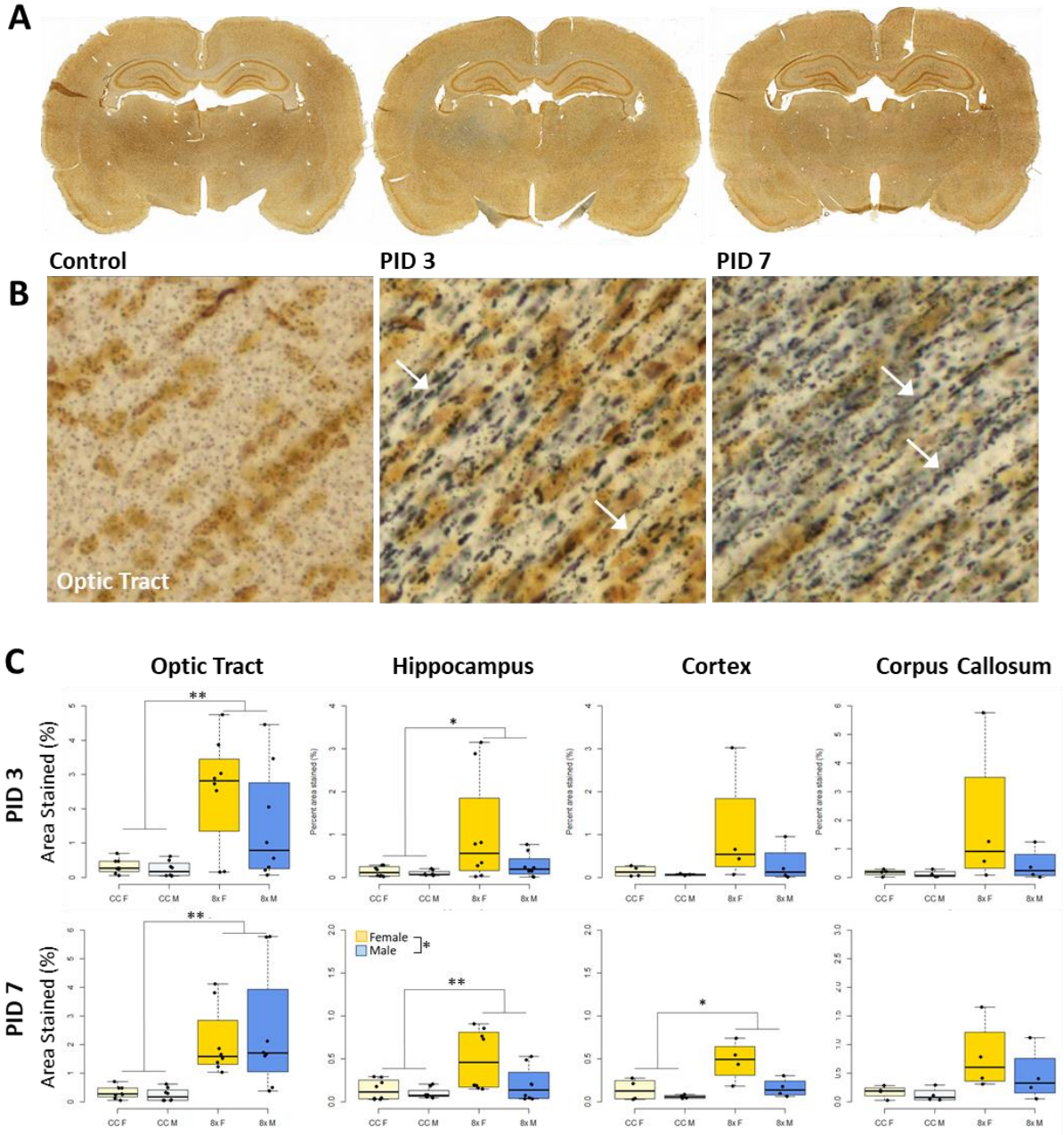

Figure 4.10: FD NeuroSilver™ stain uptake was increased after 8xACHI. Neurodegeneration was assessed using FD NeuroSilver II histology in four ROIs: the bilateral optic tract, and hippocampus; the ipsilateral cortex; and the corpus callosum. A positive stain appears as a dark grey to black particulate predominantly in the axons of degenerating cells. The percentage of the area stained in each ROI was measured. **(A)** Representative images show a coronal section from the control, 8xACHI on PID3, and 8xACHI on PID7 groups (100x magnification; 10x objective). **(B)** Representative images were selected ad hoc to show silver stain deposition. They are sampled from 200x magnification micrographs, but an additional 300% digital zoom was applied to better visualise stain uptake patterns. Linear aggregation in white matter tracts and not overlapping with cellular nuclei suggest stain uptake might be associated with axons **(↘)** (200x magnification; 20x objective; additional 300% digital zoom) **(C)** The 8xACHI group had a larger percentage of the ROI stained in the optic tract on PID 3 (**), and on PID7 (**). The 8x ACHI group also had a significantly larger area stained in the hippocampus on PID 3 (*) and PID 7 (*), and in the cortex on PID 7 (*). There was a significant main effect of sex in the hippocampus on PID 7(*) (*: $p<0.05$; **: $p<0.01$| ACHI awake closed head injury; CC cage control; F female; M male; PID post injury day; ROI region of interest)

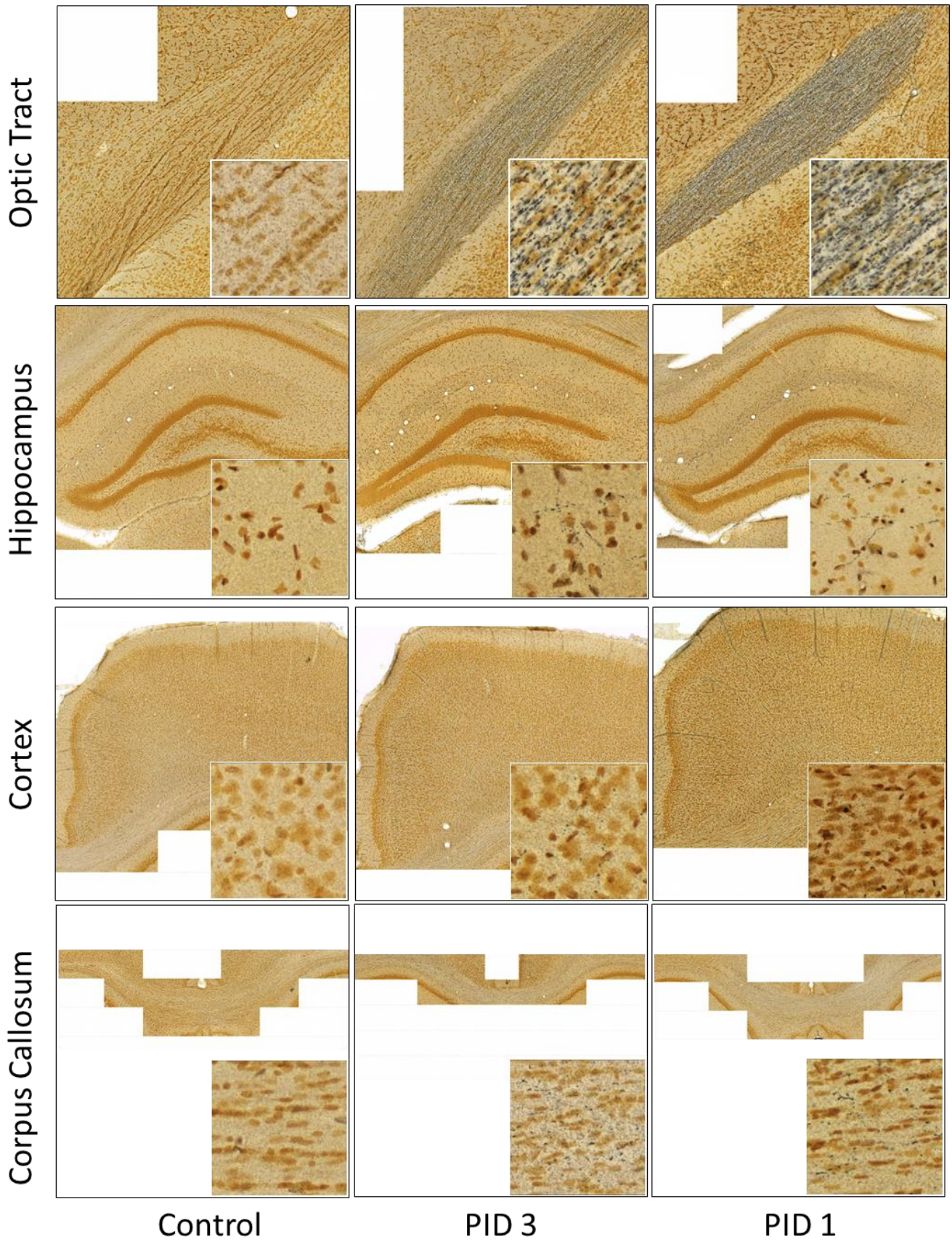
Optic Tract
Hippocampus
Cortex
Corpus Callosum
Control
PID 3
PID 1

Figure 4.11: Representative images of the four ROIs examined in FD NeuroSilver™ histology. Stain uptake was highly variable between individuals and ROIs. Grey-black granules indicate positive stain, and were most densely visible in white matter regions like the optic tract. A composite image of each ROI (one for each ROI, in every slice, in every rat) was created by combining multiple 200x magnification (20x objective) micrographs using StereoInvestigator. The images shown above are taken from these composite images. Insets are taken from the 200x magnification micrographs images, but an additional 300% digital zoom was applied to explore stain uptake patterns. In order to confirm and analyse this stain uptake localization, imaging with a higher magnification objective would be needed. (PID post-injury day; ROI region of interest)

4.4 Chapter Summary and Conclusions

The goal of this characterisation was to gain a comprehensive understanding of the injury provided by the ACHI model, particularly a reliable and reproducible subset of behavioural sequela that are analogous to clinical concussion symptoms. Establishing these characteristics will lay a crucial foundation for future investigations of the mechanisms underlying concussion symptoms, and treatments that modulate them. In these experiments, we sought to determine whether 8xACHI produces acute neurologic impairment, cognitive impairment, and neurodegeneration. Our NAP was used to assess neurologic function immediately after each injury. The Barnes maze reversal task was used to assess cognitive function in the first hour, and three days after injury. Silver stain histology was used to detect neurodegeneration on PID 3 and 7. We determined 8xACHI produced acute neurologic impairment with the NAP, and a mild spatial learning deficit potentially mediated impaired cognitive flexibility in Barnes maze reversal training. These were accompanied by neurodegeneration in the optic tract, hippocampus, and ipsilateral cortex during the first week of recovery. Diffuse neurodegeneration was observed in the hippocampus and cortex, which might contribute to learning and cognitive flexibility impairments in reversal learning. This is potentially confounded by visual impairments due to axonal injury in the optic tract. A main

caveat of our finding is that the injury timeline may not correspond well to the majority of clinical repeated concussion. Future studies should investigate more representative injury timelines. Future studies should also include more challenging behavioural tests to identify a more robust cognitive deficit, and additional histology to characterize the observed neurodegeneration. Taken together, these findings further support the utility of our novel ACHI model in preclinical concussion research.

Chapter 5 - Discussion

5.1 Summary of Objectives and Experiments

Concussions are a type of brain injury and constellation of symptoms that can result from the transmission of any biomechanical force to the brain. They represent a significant global health burden, and are therefore the subject of a growing body of medical research. Preclinical models of concussion have been used to examine pathophysiological processes underlying symptoms, which is an important step in developing new diagnostic and treatment strategies. Historically the clinical translation of concussion research has been limited. Two factors that may contribute to this are the use of anaesthesia, and tendency to focus on adult male subjects. These means of reducing variability are justified, but preclinical research moving forward should address limitations to translatability by using both sexes, including pediatric age-groups, and omitting anaesthesia where possible. To address this we developed the ACHI model; a new preclinical model for pediatric concussion. The ACHI model is well-suited to studying preclinical concussion because it allows for rotational and vertical displacement of the head, and produces a helmeted closed-head injury. It is the first of its kind in Canada to model concussion without anaesthesia. Before this model can be used to investigate concussion mechanisms and potential diagnosis or treatment methods, it must be characterized to demonstrate that it produces a set of behavioural and pathological outcomes similar to clinical concussion.

In our initial characterisation described in **Chapter 3**, we set out to show that our model recapitulates the definition of concussion provided by CIGS 5. All studies used both male and female juvenile rats. Our newly developed NAP was used to test acute neurologic function

immediately after the injury. The Barnes maze, elevated plus maze, open field test, and Rotarod were used to assess changes in cognition, anxiety, and locomotion in the first week of recovery. Finally, structural MRI was used to determine if the model produces a visible lesion, hemorrhage, or volumetric changes.

Chapter 4 summarizes additional characterisation to further investigate cognitive impairment after ACHI, and to investigate potential causes of white matter abnormalities observed in advanced MRI findings. An updated NAP was used to assess acute neurologic impairments. The Barnes maze reversal paradigm was used to identify impaired learning, memory, and cognitive flexibility. FD NeuroSilver™ histology was used to detect neurodegeneration in the optic tract, hippocampus, cortex, and corpus callosum.

In the fist week after repeated ACHI we observed acute neurologic impairment in the NAP, and sub-acute cognitive impairment including reduced cognitive flexibility in the Barnes maze. This was accompanied by normal structural neuroimaging, but histology revealed diffuse neurodegeneration, and advanced MRI found white matter abnormalities. **Table 5.1** summarises major effects of ACHI on these outcomes, and **Table 5.2** summarises major sex-differences noted throughout these experiments.

Table 5.1: Summary neurocognitive and histological characterisation of ACHI. All experiments were completed in juvenile male and female Long Evans rats. The first ACHI occurred on PND 25-26.

	ACHI	Control Group	N (total, group)	End Point	Experiment	Effects of ACHI (vs. control)	Interpretation
Chapter 3	1x; 4x	Sham	(58, 18-20) (PID 1: 30, 8-11)	Immediate PID 1	NAP	▼ NAP score in 4x and 1x No effect	Acute neurologic impairment
	4x		(36,9)	1 hour PID 1	Barnes test	▲errors finding escape	Memory impairment
					Rotarod	No effect	No motor impairment
					EPM	No effect	No anxiety-like changes
					OF	No effect	No anxiety or motor changes
	1x; 4x		(46, 7-11)	PID 1 PID 7	Structural MRI	No visible lesion; no atrophy	No severe TBI
(Wortman, Meconi, et al., 2018)	*4xACHI subset from above*			*PID 1* *PID 7*	*Advanced MRI*	*▼ FA* *Differential changes in TWI streamlines*	*White matter abnormalities*
Chapter 4	8x	Cage control	(32,8)	Immediate	NAP	▼NAP score after 8th ACHI Gradual decline from 1st to 8th	Acute neurologic impairment; graded
				1 hour	Barnes Probe	No effect	No memory impairment
				PID 2-3	Rev. Training	▲ distance to escape Trial 1	Impaired cognitive flexibility
				PID 3	Rev. Probe	No effect	No memory impairment
				PID 3	Silver stain	▲ in optic tract, hippocampus	Diffuse neurodegeneration
				PID 7		▲ in optic tract, hippocampus, cortex	

▼ *decrease;* ▲*increase*| *NAP: neurologic assessment protocol; ACHI awake closed head injury; EPM elevated plus maze; OF open field maze; PID post-injury day; Rev. reversal; TBI traumatic brain injury;*

Table 5.2: Summary of sex-differences during ACHI characterisation. All experiments were completed in juvenile male and female Long Evans rats. The first ACHI occurred on PND 25-26.

	ACHI	Control Group	N (total, group)	Time Point	Experiment	Sex-differences	Interpretation
Chapter 3	1x; 4x	Sham	(58, 18-20) (PID 1: 30, 8-11)	Immediate PID 1	NAP	No effect Low score correlated with Barnes impairment in females	Increased impairment in subset of females NAP may predict cognitive outcomes
	4x		(36,9)	1 hour PID 1	Barnes test	▲errors in females ▲speed in females ▲% time moving in females	Sex-difference in spatial memory and motility
					Rotarod	No effect	No motor differences
					EPM	▼ time in open arm in females	Sex-difference in anxiety
					OF	No effect	No sex-differences in anxiety or motility
	1x; 4x		(46, 7-11)	PID 1 PID 7	Structural MRI	No effect	No sex differences in lesion or atrophy
Chapter 4	8x	Cage control	(32,8)	Immediate	NAP	▼ score in males across 8 injuries; no effect on 8th injury alone	Sex*injury interaction in NAP; more impairment in males
				1 hour PID 2-3 PID 3	Barnes Probe Rev. Training Rev. Probe	No effect	No sex differences in cognition
				PID 3 PID 7	Silver stain	▲ in hippocampus in females	Sex difference in hippocampal silver deposition

▼ *decrease;* ▲*increase*| *NAP: neurologic assessment protocol; ACHI awake closed head injury; EPM elevated plus maze; NA not analysed; OF open field; TBI traumatic brain injury; PID post-injury day*

5.2 Functional Outcomes: Neurocognitive impairment in the first week of recovery

5.2.1 A subset of rats lost consciousness after ACHI

In both the 4xACHI and 8xACHI cohorts, only a small subset of injured rats lost consciousness, and these were predominantly rats in the repeated ACHI group rather than single injury. This was demonstrated by a delayed latency to display toe pinch and self righting reflex. These findings are in agreement with clinical observations, as well as those from similar models (Castile et al., 2012; Erlanger, 2015; Marshall et al., 2015; McCrory et al., 2013; A. Petraglia et al., 2014). The observed LOC was comparable to that seen in high school and collegiate athletes, where only 5-9% of concussions produced LOC[9,10] with a median duration of 5 seconds, and with 91% regaining consciousness in less than 30 seconds(Marshall et al., 2015). Importantly, this agrees with clinical trends, as the Consensus Statement for Concussion in Sport (McCrory, Meeuwisse, et al., 2017), and Canadian guideline on Concussion in Sport (Parachute, 2017) notes that most concussions are not accompanied by LOC, (Ellis et al., 2019). Notably, apnea was never observed after ACHI, but has been reported in some anesthetized preclinical concussion models. This might reflect increased severity of those models, or support the notion that anaesthesia alters concussion outcomes.

5.2.2 8xACHI resulted in acute neurologic impairment

When a concussion is suspected in clinical cases, acute tests of neurologic function such as the SCAT5 (Petit et al., 2020), Child SCAT5 (Davis et al., 2017), or CRT5 (Echemendia, Meeuwisse, McCrory, Davis, Putukian, Leddy, Makdissi, Sullivan, Broglio, Raftery, Schneider, Kissick, McCrea, Dvorak, Sills, Aubry, Engebretsen, Lossemore, Fuller, Kutcher, Ellenbogen,

Guskiewicz, Patricios, & Herring, 2017) can be used to identify potential signs of a concussion. These tests do not provide a comprehensive or definitive diagnosis, but are useful because they can be rapidly administered by a trained medical professional, without extensive apparatus. Our NAP is modelled after elements of these tests that assess motor and reflexive changes, and has been used successfully to identify neurologic impairment in juvenile rodents after ACHI (Christie et al., 2019b; Pinar, Trivino-Paredes, Perreault, & Christie, 2020).

When comparing sham, single, and 4xACHI a trend of graded symptom severity was observed in NAP findings where a repeated injury produced poorer performance than single ACHI. Overall, these results reflect a common trend in preclinical and clinical concussion research showing that repeated injuries tend to result in more severe neurologic impairments (Guskiewicz et al., 2003; McCrea et al., 2003; McCrory et al., 2013; Anthony L. Petraglia et al., 2014; Shultz et al., 2012). In the Chapter 4 cohort, 8xACHI significantly impaired NAP performance compared to cage control. At baseline, both groups performed well, with average scores near the maximum possible. Cage controls continued to score consistently well after each trial. Scores in the 8xACHI groups declined most notably after the first injury, and continued to decline moderately through later trials. Taken together, this suggests that 8xACHI produces acute neurologic changes. This corroborates the graded decline seen in the previous cohort, and other rodent TBI models that measure acute neurologic impairment with similar tests (Dhananjay R. Namjoshi et al., 2017; Dhananjay R Namjoshi et al., 2014; A L Petraglia et al., 2014; Pham et al., 2019).

These data indicate our NAP score is a useful tool because it provides a simple, yet sensitive, assessment of neurologic function that can be rapidly applied in the diagnostically important time window immediately after the injury. However, it is limited in that it does not address more subtle cognitive, motor, and emotional changes that are common symptoms of clinical concussion (Carroll et al., 2004; Faul et al., 2010; Gibb & Kolb, 1998; McCrea et al., 2009; McCrory et al., 2013). Notably, this is analogous to the short lived neurologic impairment denoted in the CISG 5 concussion diagnostic criteria (McCrory, Meeuwisse, et al., 2017).

5.2.3 More errors were made in the Barnes maze test after 4xACHI

Learning and memory impairment, and executive dysfunction are common cognitive symptoms of clinical concussion (Ellis et al., 2019; Green, Keightley, Lobaugh, Dawson, & Mihailidis, 2018; Sady, Vaughan, & Gioia, 2011). The Barnes maze is a behavioural test of learning and memory function in rodents (Barnes, 1979; Rosenfeld & Ferguson, 2014). It has been variably adapted for use in juvenile rats (McHail, Valibeigi, & Dumas, 2018; Valibeigi, McHail, Kimball, & Dumas, 2018), and has been previously used to identify spatial learning and memory deficits in preclinical TBI models (Fedor, Berman, Muizelaar, & Lyeth, 2010; McAteer, Corrigan, Thornton, Turner, & Vink, 2016; Mouzon et al., 2012). In the Chapter 3 cohort, the 4x ACHI group made significantly more errors compared to sham animals in the Barnes maze test 1-hour after final injury, indicating impaired memory. This is analogous to the common complaint of memory problems after concussion. This deficit was not observed on PID 1, and a convergent measurement of memory (i.e. distance to escape) in the same trial did not support this finding, which suggests memory impairments caused by 4xACHI are subtle and transient. A review of Barnes maze outcome measures found that time in target quadrant during a probe

trial is a superior metric for memory impairment in the Barnes maze (O'Leary & Brown, 2013). In the Chapter 4 cohort, a probe trial with the escape box removed was used. In this cohort, no memory impairments were found 1-hour after 8xACHI. It was hypothesized that the 8x injury repeat number would create a more severe injury phenotype than the 4x repeat number, thus the absence of spatial memory deficits in the Barns maze probe does not agree with our initial findings. It is possible that the disparities in our findings of memory impairment are due to the differences in the probe test method used for each cohort. A classic probe test was used in the Chapter 4 acquisition and reversal probe trials, where the escape box was removed and the rat was allowed to freely explore the maze for 90 seconds. In the Chapter 3 cohort, an alternative style of probe was used in order to prevent extinction of the memory of the escape hole location between the 1-hour and PID1 tests. Another factor which may have contributed to disparities in Barnes maze acquisition outcomes is that the cohort for Chapter 4 were primarily shipped cross-country as weanlings, whereas the cohort used in Chapter 3 was primarily bred in house. Travelling can be a stressful experience for rodents with physiological effects of stress such as elevated plasma corticosterone lasting for up to two weeks after arrival (Arts, Kramer, Arndt, & Ohl, 2012). It is possible that behavioural effects of early-life stress exposure masked behavioural effects of ACHI.

5.2.4 Barnes maze reversal learning was impaired after 8xACHI

Previously we have shown that four repeated ACHIs produced mild learning impairments in the Barnes maze task in females (Meconi et al., 2018). We hypothesized that increasing the injury number and task complexity would produce a greater deficit in Barnes

maze performance. To test this, we adjusted our paradigm to include the Barnes maze reversal task (O'Leary & Brown, 2013).

8xACHI did not affect performance in either probe trial, but the 8xACHI group took longer to locate the escape hole in the first reversal training trial. This is indicative of impaired learning, but the reversal task also requires cognitive flexibility (Barnes, 1979; Crews & Boettiger, 2009; Gawel et al., 2019). It is possible the reversal learning impairment we observed is mediated by impaired cognitive flexibility. The deficit recovered rapidly, as both groups performed equivalently in the remaining reversal training trails. Given that cognitive impairments can persist for one to four weeks in clinical cases (Tracey Covassin et al., 2010; Holmes et al., 2020; McCrory, Meeuwisse, et al., 2017; Nance et al., 2009), we had hypothesized that 8xACHI would produce more persistent learning impairment throughout the reversal training, and memory deficits in probe trials. Nonetheless, impaired learning and cognitive flexibility in Barnes maze reversal learning are analogous to concussion symptoms Additional behavioural experiments may be needed to identify persistent cognitive deficits that are similar to clinical symptoms.

5.2.5 NAP scores variably predicted cognitive deficits

In clinical cases, the SCAT5, child SCAT5, and CRT are popular standardized tool used by physicians to help diagnose concussions. They can be used immediately after a suspected concussion has occurred, and to monitor recovery (Chin, Nelson, Barr, McCrory, & McCrea, 2016; Davis et al., 2017; Echemendia, Meeuwisse, McCrory, Davis, Putukian, Leddy, Makdissi, Sullivan, Broglio, Raftery, Schneider, Kissick, McCrea, Dvorak, Sills, Aubry, Engebretsen, Lossemore, Fuller, Kutcher, Ellenbogen, Guskiewicz, Patricios, Herring, et al., 2017). Initial

SCAT performance may be predictive of the duration of recovery and severity of symptoms (Tkachenko, Singh, Hasanaj, Serrano, & Kothare, 2016), and thus helpful for planning individualized treatment strategies. Behavioural outcomes can be compared to NAP score results to determine whether the NAP score may be predictive of the duration and complexity of symptoms in this model. In the Chapter 3 cohort, we found a moderate correlation between low NAP scores and poor Barnes maze performance in females after 4xACHI. In the Chapter 4 cohort NAP score broadly did not correlate with Barnes maze reversal or probe impairments. This loss of predictive capacity might be related to the expanded scoring method used in the Chapter 4 cohort. Although a simple linear regression was a suitable choice for a preliminary examination of this potential relationship, it is possible a more sophisticated regression analysis is needed to properly model the relationships between NAP scores and behavioural outcomes, if they correlate.

An important goal for clinical concussion research is to better understand the extensive heterogeneity in concussion symptoms and recovery. This has been linked to risk factors that were present before the injury, and the nature of the injury itself (Rosenbaum & Lipton, 2012). This problem may be investigated using models like ACHI by identifying pathophysiological differences between subsets of rats that experience the most severe symptoms, and the ones that experience few or no symptoms, despite having received the same head impact. Our NAP had limited predictive value in the severity of cognitive changes. Nonetheless we demonstrated a subset of rats experienced more severe symptoms. This is seen in clinical trends as well. Future work should use a similar approach to identify subsets of more severely impaired rats, and then investigate subtle physiologic differences between those that develop mild and severe

symptoms in response to the same ACHI. This may provide insight on why symptomology is so diverse in clinical cases.

5.2.6 Anxiety and motor function were not affected by repeat ACHI

No changes in anxiety-like behaviours were observed in the elevated plus maze or open field after repeated ACHI. Similarly, repeated ACHI did not produce differences in the latency to fall from the Rotarod, or in measurements of speed and distance travelled in any of the mazes. Motor deficits and increased anxiety are both common symptoms of clinical concussion (Ellis et al., 2019, 2015). Given that impairments after clinical concussion can be transient and subtle (A. D. Wright et al., 2017), and can emerge over time (McCrory et al., 2013; Morgan et al., 2015), future work should extend the post-injury timeline. There are limitations to the ability to replicate human conditions in rodents, and it is possible rodents are not suitable candidates to model the complex cognitive processes mediating anxiety after concussion. It is also possible that the elevated plus maze and open field were not sensitive to subtle changes in anxiety related to ACHI. Future work may require more sensitive behavioural assessments to explore how repeated ACHI affects anxiety.

5.2.7 Sex-based differences in behavioural outcomes

The correlative comparison of NAP scores and Barnes maze outcomes revealed a subset of female rats that performed much worse than their injured and uninjured counterparts alike. Some clinical studies have found that concussion symptoms in females tend to be more severe and longer in duration (Bazarian, Blyth, Mookerjee, He, & McDermott, 2010; Tracey Covassin, Elbin, Harris, et al., 2012; Tracey Covassin, Elbin, Larson, et al., 2012; Tracey Covassin et al., 2016; McCrory et al., 2013), although some experimental models find the opposite, supported by

the neuroprotective effects of estrogen (Bramlett & Dietrich, 2001; R L Roof & Hall, 2000). In the Chapter 4 cohort, there were no sex differences in NAP score after the final injury, but when all eight NAP scores are considered together, the males performed significantly worse than the females. That only a subset of 4xACHI females had more severe symptoms, and that a single NAP measurement missed the trend of males performing worse than females across all NAP trials, suggests sex differences in symptoms may be subtle, transient, and require more sensitive tests to elucidate. Recently we have shown that measures of synaptic plasticity may help to elucidate functional sex differences following a closed head injury by a weight drop model in anaesthetized animals (White et al., 2017), and similar examinations may offer the veracity needed to adequately explore this issue following single and repeated ACHI. Notably, at this age rats are pre- or peri-pubescent. Sexual dimorphisms such as differences in weight and external sexual organs are just becoming apparent over the timeline of these experiments. It is possible that dimorphisms mediating sex-differences in outcomes have yet to emerge at this age. However, we observed significant difference between males and females in several behavioural tasks.

In Chapter 3, females made more errors than males in the Barnes maze probe trial. They also moved significantly faster and spent a greater percentage of time moving than males. In the same cohort, females also spent significantly less time in the open arm of the elevated plus maze; an indication of increased anxiety.

Barnes maze acquisition training happened before receiving ACHI. There was a significant interaction of injury group and sex, where males in the 8xACHI group performed worse in males in cage control group. This was mediated by an apparently anomalously poor

performance by a subset of males during the 5th trial. However, a non-significant trend of males performing worse was observed in the 5th trial of the Chapter 3 cohort, as well. Notably, the 5th trial was the first after an overnight break for both training schedules. It appears that in this Barnes acquisition paradigm, juvenile male rats struggle to recall the escape-hole location in the first trial, but perform equivalently to females in subsequent trials that day. This may reflect a sexual dimorphism in cognitive development.

5.3 Structural Outcomes: Diffuse neurodegeneration with no structural MRI abnormalities

5.3.1 Structural neuroimaging abnormalities were absent after ACHI

The CIGS 5 (2017) definition of concussion states that concussion symptoms result from a functional disturbance rather than a structural injury, but it is important to note that this refers to a structural injury on a macroscopic scale. Particularly that concussions do not result in abnormalities on standard structural neuroimaging like CT or MRI. The CIGS 5 (2017) emphasizes that it remains unknown whether concussions result from reversible physiological changes, or represent lesser degrees of the diffuse structural damage seen in more severe TBIs. One study noted changes in the volume of cortical structures in young athletes with a history of repeat concussion (List et al., 2015). With structural MRI we found that *ex vivo* whole bran scanning revealed no reduction in the volume of the hippocampus, cortex, or corpus callosum, indicating that single and 4xACHI did not produce overt structural damage in the first week of recovery.

Advanced neuroimaging found white matter abnormalities after repeated ACHI

An advanced MRI study using diffusion weighted imaging (DWI) was conducted using the same brains from the structural MRI analysis (Wortman et al., 2018). Because the findings are from the same cohort, they relevant in characterising ACHI outcomes. DWI is a promising diagnostic approach that can be used to identify white matter abnormalities resulting from single or repeat concussion(Shenton et al., 2012; D. K. Wright, Trezise, et al., 2016). Similar to clinical findings, we observed reductions in FA, and TWI changes. Interestingly, only TWI distinguished between control and repeat ACHI on PID 1. This suggests TWI is a potential early diagnostic method. It is noteworthy that repeat ACHI reduced FA, in light of the cognitive flexibility impairment we observed in the Barnes maze reversal task, since FA reductions predicted executive dysfunction in a clinical concussion study (Miles et al., 2008).

Diffusion tensor imaging (DTI) is a common method of DWI, which has been used to identify white matter changes including diffuse axonal injury after clinical concussion (Inglese et al., 2005). Fractional anisotropy (FA) is a DTI metric that is reduced after clinical concussion (Bazarian et al., 2007; Niogi et al., 2008; Wilde et al., 2008). Track weighted imaging (TWI) is a newer method of DWI that may be more sensitive to white matter pathology (Calamante et al., 2012; Pannek et al., 2011). Other preclinical concussion models have reported TWI changes as well (D. K. Wright et al., 2018; D. K. Wright, Trezise, et al., 2016). Taken together, this suggests our novel ACHI model produces microscopic white matter abnormalities that do not appear in typical structural neuroimaging.

5.3.2 FD NeuroSilver™ histology showed diffuse neurodegeneration after 8xACHI

Previously, we observed acute neurologic and behavioural impairment in the absence of typical neuroimaging abnormalities (Meconi et al., 2018). Advanced neuroimaging in the same brains identified white matter abnormalities, which might indicate diffuse microscopic axonal injury. We hypothesized that repeat ACHI can induce diffuse microscopic cellular damage producing neurodegeneration. Other preclinical TBI models have used histological approaches to detect neurodegeneration after single and repeat injury (Dhananjay R. Namjoshi et al., 2017). In particular the NeuroSilver™ II (FD NeuroTechnologies, Columbia, MD) staining method, which detects degenerating neuronal somas, axons, and terminals, is an established method to detect neurodegeneration in preclinical concussion models (Evanson, Guilhaume-Correa, Herman, & Goodman, 2018; Dhananjay R Namjoshi et al., 2014). In agreement with neurodegeneration observed in other preclinical concussion models, we found a significant increase the silver stained area of the optic tract, hippocampus, and cortex in the first weak after 8xACHI.

A study using silver stain to detect neurodegeneration after experimental concussion used higher magnification (i.e. 100x objective) images to conclude that axons are a primary site of damage. They found punctate patterns of stain uptake associated with fibers in multiple white matter tracts (Dhananjay R Namjoshi et al., 2014). They also noticed axonal varicosities, which are a pathological hallmark of clinical TBI (Johnson et al., 2013). An *ad hoc* examination of our images found punctate uptake pattern, as well as linear aggregates of stain that suggest axonal uptake **(Fig. 5B)**. In the future, imaging with a higher magnification objective would help

to confirm the presence of axonal varicosities. Our previous MRI DWI found white matter abnormalities, and our silver stain experiment has detected diffuse neurodegeneration in white matter (i.e. the optic tract) and in regions with both white and grey matter (i.e. the cortex and hippocampus) Taken together, this suggests diffuse neurodegeneration including axonal injury are pathological outcomes of repeat ACHI.

Tissue that has been processed with the NeuroSilver™ II (FD NeuroTechnologies, Columbia, MD) method may also be used for immunohistochemical staining. Future studies may use immunohistochemical techniques to characterize regions with elevated silver uptake. Knowing what type of cells are degenerating, whether the degeneration represents necrotic or apoptotic cell death, and whether neuroinflammation is increased in these areas will provide useful pathophysiological insights.

5.4 Limitations and Future Directions

A main advantage of the ACHI model and NAP scoring is that it allows the researcher to measure the rapid evolution of changes immediately after injury, without the potential confounds of surgical or anaesthetic recovery. Anaesthesia is known to have neuroprotective effects (Hendrich et al., 2001; Statler, Alexander, Vagni, Dixon, et al., 2006; Statler, Alexander, Vagni, Holubkov, et al., 2006), and it is important to acknowledge that this has the potential to affect outcomes in models where it is used. A primary focus of future experiments is to explore whether acute anaesthetic exposure at the lower dosages commonly used in rodent models of mTBI may be a potential confounder.

An important caveat to address in future experiments investigating repeated ACHI is the timeline of injuries. Our timeline is clinically relevant, as has been demonstrated by a recent quantification of impact exposure in football players, which found they experience an average of 6.3 impacts per practice, and 14.3 impacts per game Crisco et al., 2010), which is greater than the four- or eight-injury paradigms used here. Some sport (Crisco et al., 2010; Rutherford, Stephens, Potter, & Fernie, 2005; Terwilliger, Pratson, Vaughan, & Gioia, 2016), or military (MacGregor, Dougherty, Morrison, Quinn, & Galarneau, 2011) exposures can lead to multiple concussions within several hours or days, comparable to our timeline, but the majority of clinical repeat concussions have several months to years between injuries (MacGregor et al., 2011; McCrea, Broglio, McAllister, Zhou, et al., 2020). Furthermore, a shorter interval between injuries is associated with poorer outcomes (Eisenberg, Andrea, Meehan, & Mannix, 2013). Therefore, a longer interval between ACHIs might be more representative of clinical repeat concussion, and should be considered in future experiments.

The Barnes maze reversal task requires hippocampal and prefrontal processing (Barnes, 1979; Harrison et al., 2006), and the neurodegeneration we observed in these regions might have contributed to the reversal learning impairment. Our ability to draw functional connections here is limited because the section of cortex we studied only partially overlaps with prefrontal cortex. Additionally, the reversal impairment occurred on PID 2, and cortical neurodegeneration was not observed until PID 7, meaning there was no direct temporal overlap. It is possible underlying neurometabolic pathophysiology (Christopher C Giza & Hovda, 2014) initiated by 8xACHI contributed to acute prefrontal impairment in the reversal task, and to the diffuse neurodegeneration we observed.

The most notable neurodegeneration was observed in the optic tract. This may be clinically relevant, and warrants further investigation in two ways. Firstly, visual impairment is a common clinical concussion symptom (Ellis et al., 2019), and the neurodegeneration we found in the optic tract could suggest 8xACHI could have affected vision. Since navigation in the Barnes maze is vision-based (Rosenfeld & Ferguson, 2014), it is possible visual impairment affected performance rather than impaired cognitive flexibility or learning. This could be addressed in future Barnes maze paradigms with a cued probe trial where the escape is marked by a proximal visual cue. The validity of such visual cued trials in the Barnes maze is questionable, as it has been shown that they do not reliably differentiate visual versus spatial deficits because rodents prefer a spatial navigational method based on distal cues even when a proximal cue is available (Harrison et al., 2006). Secondly, the observed optic tract damage may be relevant to the clinical observation of optic neuropathy, which is associated with several neurodegenerative diseases including Alzheimer's and Parkinson's (Carelli, Morgia, Ross-Cisneros, & Sadun, 2017). Optic neuropathies appear to are associated with the optic nerve being particularly vulnerable to damage due to mitochondrial dysfunction. In repetition of FD NeuroSilver™ histology after ACHI, the optic nerve should be examined as well, to determine whether a similar extent of degeneration occurs in the optic nerve as was observed in the optic tract. This may represent a promising diagnostic avenue for concussion, as diagnostic tools to aid in the detection of optic neuropathies already exist.

Cognitive changes after clinical concussion are often subtle, and involve impairment to tasks requiring higher levels of executive function (Tracey Covassin et al., 2010; Holmes et al., 2020). These can be difficult to replicate in rodent models, and behavioural tasks that are too

simple may fail to identify more subtle cognitive deficits relevant to symptoms associated with clinical concussion. In fact, in a recent study where 8xACHI were administered over a longer timeline of four days instead of two, we found no cognitive impairments in the Barnes maze reversal task, but found that the repeat injury group had impaired performance in the novel object location task (Pinar et al., 2020). This suggests that novel context recognition tasks, which are spatial tests for object location memory (Haettig et al., 2011; Mumby, 2002), might be more useful in future experiments identifying subtle cognitive deficits caused by ACHI. The early cognitive impairment we observed in the reversal task recovered rapidly. All rats appeared to have learned the new location by the second trail, as there was no change in performance in the following four trials. This suggests fewer training trials could be used in future experiments using this Barnes maze reversal paradigm.

It is notable that there appeared to be increased variability in behavioural and histological outcomes after ACHI, where some rats showed much more severe impairment or damage than controls, but others were no different. It is possible that this variability in outcomes is evidence that the ACHI device, or researcher, were not providing a consistent injury between subjects, but this extensive variability is also seen in clinical populations. It appears that the location and absolute magnitude of force of impact alone does not predict the severity of damage, but individual physiological differences also exist that can modulate symptom severity under biomechanically similar impact conditions. That is, there are individual differences in the threshold of force an impact must reach in order to produce concussion symptoms (Rowson et al., 2019). In the context of pre-clinical research, this may be interpreted to support the idea of increasing group sizes in order to allow for the identification

of statistically relevant sub-populations of rats showing more or less severe symptoms and pathologies after ACHI. Comparing pathophysiologies between such groups, as well as controls, may provide insight on physiological vulnerabilities to concussion.

Similarly, ACHI and behavioural tests in these experiments occur at a peripubertal time (PND 28-30) corresponding with the onset of estrus in female rats(Ojeda & Andrews, 1981). In rats, the estrus cycle spans four days, and is characterised by fluctuating levels of estrogen and progesterone. Clinical and pre-clinical research suggests estrogen can be neuroprotective (Simpkins & Singh, 2008), as can progesterone (Robin L. Roof & Hall, 2000). However, a more rapid decline of progesterone during the luteal phase is associated with more severe pre-menstrual symptoms like headache, fatigue, and mood disturbance (Lovick et al., 2017). These are notably also common symptoms of concussion. Female athletes who sustained a concussion were more likely to be in the late luteal phase of the estrus cycle at the time of injury(La Fountaine et al., 2019; Wunderle, Hoeger, Wasserman, & Bazarian, 2014). It is possible that women are vulnerable to a more severe injury during the late luteal phase because the decline in progesterone reduces neuroprotection, or because the concurrent pre-menstrual symptoms during this phase exacerbate concussion symptoms to a detectable threshold. Future research using the ACHI model in females should monitor the estrus cycle, and determine whether symptom severity correlates with estrus phase during ACHI or testing. It is possible that the progesterone decline which occurs during pro-estrus in rodents may affect the severity of symptoms, similar to the progesterone decline that occurs during the late luteal phase in humans. If being in a certain phase of the estrus cycle can predispose an individual to more severe symptoms, this may represent a separate sub-group for experimental assessment. If so,

this would warrant increasing the number of females used so that the data are sufficiently powered for appropriate statistical analysis. Experimental manipulation of circulating hormones during ACHI may help to elucidate whether the estrus cycle affects behavioural outcomes. For example, ovarectomy and estradiol supplementation can be used to manipulate circulating estrogen, and progesterone effects can be manipulated through administration of progesterone, or progesterone receptor antagonists.

As a new platform for concussion research, potential future directions for the ACHI model are extensive. Beginning with ever-improved characterisation, moving on to explore mechanistic insights, ultimately aiming towards developing and testing new diagnostic and treatment technologies.

5.5 Conclusions

This is the first preclinical concussion model to study repeated injury in both male and female juvenile rats without anaesthesia. The ultimate goal of this characterisation was to gain a comprehensive understanding of the injury provided by this model, particularly a reliable and reproducible subset of behavioural sequela that are analogous to clinical concussion symptoms. Establishing these characteristics is crucial for future investigations into the mechanisms underlying concussion symptoms, and treatments that modulate them. Our NAP showed acute neurologic changes after ACHI. The Barnes maze and reversal test demonstrated mild memory impairment; and impaired cognitive flexibility during reversal learning. Silver stain histology, advanced MRI, and structural MRI demonstrated diffuse neurodegeneration and white matter abnormalities in the absence of visible lesion or atrophy. Taken together these observations reflect all four of the criteria that define a concussion according to CISG 5 (McCrory,

Meeuwisse, et al., 2017). We have shown that 1) an "impulsive" force transmitted to the head results in 2) the rapid onset of short-lived neurologic impairment that resolves spontaneously. This occurs 3) with normal structural neuroimaging, and 4) produces cognitive impairment, and LOC in a subset of cases. Diffuse neurodegeneration was observed in the hippocampus and cortex, which might contribute to learning and cognitive flexibility impairments in reversal learning. This is potentially confounded by visual impairments due to axonal injury in the optic tract. A main caveat of our finding is that the injury timeline may not correspond well to clinical concussion. Future studies should investigate clinically-relevant injury timelines, behavioural assessment of more subtle cognitive abnormalities, and additional histology to characterize the observed neurodegeneration. Taken together, these findings demonstrate that ACHI is a clinically relevant model of pediatric concussion.

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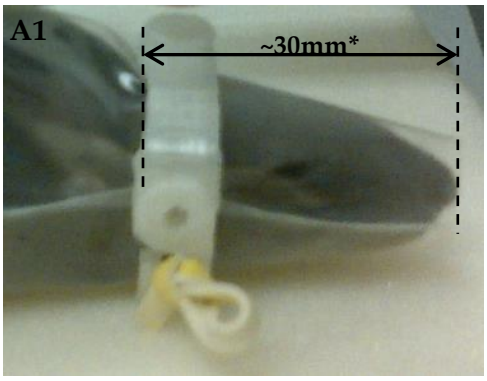
Appendix A: ACHI pilot to assess risk of skull fracture

Summary: As a requirement for initial approval by our animal care committee, we were required to perform a small pilot in which we demonstrated that the impact parameters we used did not result in skull fracture. Impact parameters that were derived from similar models were tested in euthanized animals. All apparatus and parameters used in these tests are identical to those used in experimental trials.

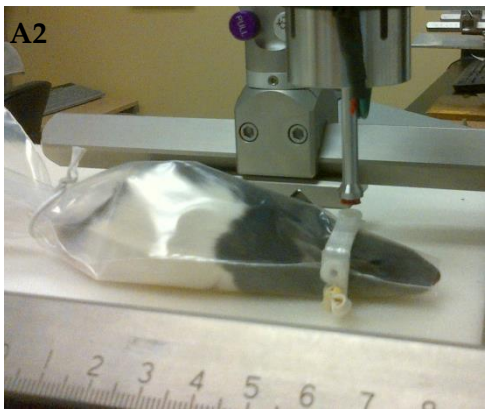
Conclusion: It was determined that an impact velocity of 6m/s, a dwell time of 0.01s, and an impact depth of 10mm were sufficient to cause a rapid acceleration of the rat's head but did not produce skull fracture.

Procedure:

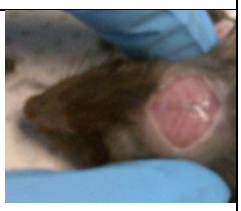
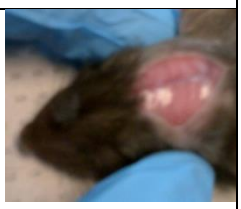
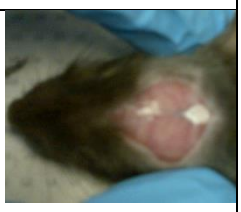
1. Rat was euthanized using anesthetic overdose (>5% isoflurane in bell jar).
2. Rat was weighed
3. Rat was placed in a conical restraint bag such that the nostrils were free of plastic and protruded from the open tip. The animal's position in the bag was adjusted so that limbs were aligned neutrally under the body.
4. The restraint bag was tied loosely around the base of the rat's tail with a twist tie.
5. The helmet was placed on the rat's head and secured with a rubber band. Helmet placement was guided using visible landmarks. The helmet was centered over the head, with the impact site over the left hemisphere. The caudal edge of the helmet was positioned approximately 1mm rostral of the interaural line, and approximately 30mm from the nostrils (Fig. 1).



6. The rat was placed in the center of the foam platform, and the impactor was aligned with the impact site on the helmet (Fig. A2).
7. Impact parameters were verified, and impact was initiated
8. The helmet and restraint were removed, and the animals scalp was shaved.
9. A 15mm incision was made along the midline of the scalp beginning at the interaural line and extending rostrally.
10. The skull was thoroughly examined through the incision to determine whether skull fracture occurred.



11. **Results:**

Animal / Age	Sex	Weight (g)	Helmet Placement* (mm)	Impact Parameters**			Skull Fracture	Image
				Velocity (m/s)	Depth (mm)	Dwell time (s)		
Rat; P26	F	67.6	33	6	10	0.01	No	
Rat; P26	F	64.0	32	6	10	0.01	No	
Rat; P26	F	70.6	34	6	10	0.01	No	

** Helmet placement value refers to distance between tip of nostril and caudal edge of helmet*

*** Definition of impact parameters: Velocity refers to speed of impactor tip; Depth refers to distance impactor tip extends beyond impact origin (impact surface of helmet); Dwell time refers to length of time impactor remains at full extension before it is retracted.*