Novel Insights into Mild Traumatic Brain Injury Using a Clinically Relevant Rat Model

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Doctor of Philosophy

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Contents

Abstract

Mild traumatic brain injury (mTBI) is a major public health concern. Nonetheless, mTBI neurobiology is still poorly understood, and consequently there are no established biomarkers and treatments to assist clinical management and improve patient outcomes. Although rodent mTBI models enable important insights not possible in humans, the clinical relevance of many have been questioned. As such, the first experiment of this thesis characterised a novel awake closed-head injury (ACHI) in rats that avoids use of confounding anaesthesia and surgical procedures. A single ACHI induced sensorimotor, exploratory behaviour and spatial memory alterations lasting 24-48 hours reflective of clinical outcomes, along with transient glial disturbances at 72 h. The second study aimed to use the ACHI model to improve understanding of the potential neurobiological and neurobehavioural effects of repeated mTBIs. I found evidence of acute cognitive and prolonged sensorimotor impairment in rats given repeated ACHI, and that these rats also had robust sub-acute increases in serum levels of the emerging axonal injury biomarker candidate, neurofilament light (NfL). Advanced MRI analyses revealed chronic white matter changes in repeated ACHI rats, however restoration of serum NfL levels indicated a lack of ongoing axonal damage at this chronic stage. Using mass spectrometry, I found novel proteomic changes in the hippocampus of repeated ACHI rats. Given many of the altered proteins observed where related to energy metabolism, my final study investigated changes to hippocampal metabolites after single and repeated ACHI using the potential biomarker, proton magnetic resonance spectroscopy (^{1}H-MRS) and investigated whether these changes were due to alterations in mitochondrial bioenergetics. ^{1}H-MRS revealed alterations in some metabolites after repeated ACHI that were not accompanied by mitochondrial dysfunction. Taken together, this thesis introduces a new pre-clinical model of mTBI, and contributes new findings that improve our understanding of the neurobiology underlying this potentially devastating condition.

Statement of Authorship

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution. All research procedures reported in the thesis were approved by the relevant Ethics Committee, Safety Committee or authorised officer.

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Abbreviations

%	Percentage
<	Less than
=	Equal
>	Greater than
+	Plus
±	Plus minus
~	Approximately
°	Degrees
°C	Degrees Celsius
3D	3-Dimentional
α	Alpha
AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Beta-amyloid precursor protein
ATP	Adenosine triphosphate
ACHI	Awake closed-head injury
β	Beta
Ca^{2+}	Calcium

CC	Corpus callosum
CCI	Controlled cortical impact
CCL2	C-C Motif Chemokine Ligand 2
Cf P	Corticofugal pathways
Cho	Choline
cm	Centimetre
Cr	Creatine
CSF	Cerebral spinal fluid
CTE	Chronic traumatic encephalopathy
d	Days
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DTI	Diffusion tensor imaging
e.g.	For example
EC	External capsule
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ETC	Electron transport chain
ETF	Electron flow
EPM	Elevated plus maze

FA	Fractional anisotropy
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
Fim	Fimbria of hippocampus
FP	Fluid percussion
FPI	Fluid percussion injury
GABA	Gamma aminobutyric acid
GCS	Glasgow Coma Scale
GFAP	Glial fibrillary acidic protein
Glc	Glucose
Glu	Glutamate
Gln	Glutamine
GPC	Glycerolphosphocholine
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
^{1}H-MRS	Proton-Magnetic resonance spectroscopy
h	Hours
h-tau	Hyperphosphorylated tau
i.e.	That is
IC	Internal capsule
ICV	Increased cerebral vulnerability
Iba-1	Ionised calcium-binding adaptor molecule

IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
K^+	Potassium
kg	Kilograms
Ln	Log
LOC	Loss of consciousness
m	Meters
MAS	Mitochondrial Assay Solution
MD	Mean diffusivity
min	minutes
MRI	Magnetic resonance imaging
msec	Millisecond
mTBI	Mild traumatic brain injury
NAA	N-acetylasparate
NE	North-east
NfL	Neurofilament light
NFT	Neurofibrillary tangles
NLRP3	nucleotide-binding oligomerisation domain-like receptor pyrin domain-containing-3
NMDA	N-methyl-D-aspartate
NW	North-west

OCR	Oxygen consumption rate
OF	Open field
PBS	Phosphate-buffered saline
PCh	Phosphocholine
PCr	Phosphocreatine
PCX	Parietal cortex
PFA	Paraformaldehyde
PND	Post-natal day
PPCS	Persistent post-concussion symptoms
RCR	Respiratory control ratio
RD	Radial diffusivity
ROI	Region of interest
ROS	Reactive oxygen species
S100B	S100 calcium-binding protein B
SCAT	Sports Concussion Assessment Tool
SE	South-east
SEM	Standard error of mean
s	Seconds
SRC	Sports related concussion
State 3u	State 3 uncoupled

State 4o	State 4 oligomycin
SW	South-west
TBI	Traumatic brain injury
TBSS	Tract-based spatial statistics
TDP-43	TAR DNA-Binding protein 43
TFCE	Threshold free cluster enhancement
TNF-α	Tumour necrosis factor alpha
Tris-HCl	Tris-Hydrochloride

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Chapter 1: Literature Review

1.1 Introduction

Traumatic brain injury (TBI) is a biomechanical force to the brain, resulting in a complex cascade of pathophysiological consequences to the brain causing transient or permanent cognitive, behavioural and/or physical impairment [1, 2]. These injuries are typically categorised based on injury severity, with injuries broadly classified as mild, moderate and severe. Mild traumatic brain injuries (mTBIs), such as concussions, are the most common form of TBI, with a 10-fold higher burden on healthcare than both moderate and severe TBI [3]. Furthermore, evidence of gross underreporting indicating that true mTBI prevalence may be much higher [4]. mTBIs are particularly common in collision sports athletes and military personnel, however they are also a leading cause of emergency department visits due to slips, falls, and motor vehicle accidents. mTBI is a heterogeneous injury with a large variety of neurobehavioural (e.g. cognitive and emotional impairments) and physical (e.g. headache, sensorimotor deficits) effects. Although mTBI symptoms typically resolve within 7-10 days, persistent post-concussion symptoms (PPCS) lasting several weeks or longer, develop in approximately 15% of individuals [2, 5, 6].

While the symptoms of mTBI typically resolve spontaneously in most clinical cases, in recent years it has become increasingly evident that experiencing multiple mTBIs can have cumulative effects, including increased risk of PPCS [7], or even neurodegenerative conditions such as chronic traumatic encephalopathy (CTE) [8], Alzheimer's disease (AD) [9] and amyotrophic lateral sclerosis (ALS) [10]. It is however important to note that these associations are controversial, primarily as clinical evidence to date is reliant on case studies and retrospective analyses. Moreover, although understanding of the chronic neuropathology in conditions linked to a history of mTBIs has increased substantially over the past two decades, the neurobiological mechanisms through which repeated mTBIs may trigger such chronic neuropathology remains poorly

understood. Further research is required to provide greater understanding of these unknowns so as to improve the clinical management of individuals with at risk of experiencing multiple mTBIs.

There are major limitations (e.g. ethical issues, inadequate technology, selection biases, heterogeneity of injury) and confounding factors (e.g. comorbidities, socioeconomic/lifestyle factors) involved in studying TBI and its short- and long-term effects in humans. Animal models allow for the control of several confounding factors, as well as carefully detailed evaluation of neurobiological measures in a shorter time period (e.g. months vs. decades) [11]. Although current animal models of mTBI have created important new insights, models that require anaesthesia and/or surgical procedures such as craniotomy (removal of part of the skull) can be questioned in terms of their clinical relevance to mTBI. Anaesthetics are reported to have significant neuroprotective effects and compromise behavioural testing in the acute stages post-injury. In addition, the craniotomy procedure required for many mTBI models likely impacts intracranial pressure, disrupts vasculature, can result in significant cell loss and neurological impairment, and may be a form of mTBI in itself [12].

In this review, I will discuss the known neurobehavioural and neurophysiological consequences of single and repeated mild cerebral impacts in human and animal models, as well as analyse existing animal models and how well they reflect reported clinical mTBI symptoms and pathology.

1.2 Single mTBI and clinical symptomatology

mTBI may occur from an impact to anywhere on the body that transmits an impulsive force to the brain, with these forces thought to typically result in transient pathophysiological disturbances without overt structural damage to the brain [1]. The

high prevalence of mTBI, particularly in athletes and military personnel [2], makes this condition a serious societal health issue. In addition to being a heterogeneous injury, neurological tolerance towards biomechanical forces of mTBI are thought to be 'individual-specific', in which an individual's unique anatomical and biological factors may yield varying symptom and pathophysiological responses [13]. Therefore, clinical signs and symptoms of mTBI may vary from person to person.

Contrary to common belief, mTBI often occurs without loss of consciousness (LOC) [14, 15]. Self-reported clinical symptoms of mTBI are usually immediate in onset, but rapidly evolving and short-lived [2, 6, 16]. These signs of mTBI may include physical abnormalities (e.g. headache, visual disturbances, sensorimotor deficits etc.), neurobehavioural symptoms (e.g. cognitive and emotional impairments etc.), amnesia, postural instability, sleep/wake disturbances and fatigue [2, 6, 17].

1.2.1 Clinical diagnosis and management of mTBI

Diagnostic tools are needed to help determine if an individual has sustained a suspected mTBI. The most common clinical diagnostic tool for TBI is the modified Glasgow Coma Scale (GCS) which uses eye opening, motor and verbal responses to categorise injury severity from mild, moderate or severe [18]. However, its primary use is to identify more severe brain injury, with limited use for distinguishing between mildly and moderately concussed and non-concussed individuals. In the many professional sporting settings, significant strides have been made in developing tools (e.g. Sports concussion assessment tool; SCAT) to assist in mTBI diagnosis through recognition of the signs and symptoms of mTBI. The SCAT was developed by experts known as the Concussion in Sport Group and designed to be used by licensed medical professionals [2]. It is the most commonly used assessment tool for mTBI and has two major components; Immediate/on field assessment comprising of the GCS, checking for observable signs of concussion, memory and cervical spine assessment, and off-field assessment involving

symptom evaluation, neurological (balance) and cognitive screening (orientation and immediate memory), concentration, and delayed recall [2].

However, the SCATs reliance on self-reported symptoms creates numerous challenges. Evidence shows that patient under reporting of symptoms to avoid removal from play is extremely prevalent, resulting in premature return-to-play decisions while players are still symptomatic [19]. Similarly, a cross-sectional study in high school athletes found that out of 331 concussive events, 78.6% of participants did not report a concussive event to an authority figure leading to athletes returning to play whilst symptomatic [20]. This is especially worrying given that subsequent injuries in short succession are thought to lead to worsened outcomes [2, 21].

Furthermore, clinical management after mTBI diagnosis is also based on clinical symptoms, with no objective tools to predict those that are at high risk of complications (such as PPCS), nor to determine if/when the brain has neurobiologically 'recovered'. As such, there is an urgent need for more reliable, objective measures of mTBI, with clinical and preclinical research moving towards advanced neuroimaging techniques and fluid biomarkers as objective indicators injury and recovery.

1.3 Current understanding of neuropathology and potential biomarkers of clinical mTBI

There are a multitude of confounding factors and limitations when studying clinical mTBI, including its high heterogeneity and variability such as injury severity, biomechanical forces, temporal and spatial pathophysiology, genetics, injury resilience, pre-injury history, and clinical outcomes. This makes mTBI extremely difficult to study and monitor. Invasive procedures, which would allow researchers to understand the molecular and cellular changes in the brain or cerebrospinal fluid, are not feasible in the

vast majority of mTBI cases. As such, human studies rely on non-invasive advanced neuroimaging techniques and emerging peripheral fluid biomarkers candidates in order to provide objective indication of the nature and mechanisms of injury [2]. However, despite huge effort, the exact neurobiological and neuropathological mechanisms of how mTBI alters brain function in humans is still poorly understood [2, 22]. Nonetheless, this review will now outline the clinical evidence of neurobiological and neuropathological changes after mTBI.

1.3.1 Neurometabolic biomarkers

Disruptions in brain metabolism have been hypothesised to be key to the signs and symptoms of mTBI. For example, metabolic alterations observed via positron emission tomography (PET) in the brains of mTBI patients have been correlated with long-term attention, concentration and neuropsychological issues [23]. N-acetyl aspartate (NAA) is thought to be a promising metabolite biomarker of neuronal integrity [24]. This has been shown in numerous studies that have observed decreased cerebral levels of NAA in patients living with ALS [25], AD [26] and TBI [27]. Importantly, NAA synthesis is linked to the mitochondria and cellular ATP levels [24], with reduced NAA levels hypothesised to be a biomarker of the 'neurometabolic cascade' of mTBI [28]. However, most of the current knowledge on altered brain metabolism after mTBI is derived from animal studies and will be discussed later in this review.

Proton-Magnetic Resonance Spectroscopy (^{1}H-MRS) is a non-invasive neuroimaging technique using single or multiple voxels to measure numerous cerebral metabolites including, but not limited to, choline (Cho), creatine (Cr), glutamate (Glu), glucose (Glc) and NAA [29]. This technique has been touted as a potential tool for detecting metabolic imbalance in post-mTBI patients and informing return-to-play/duty decisions [24]. However, of the few human studies utilising ^{1}H-MRS in the context of mTBI, most consist primarily of small groups assessed at acute timepoints. Henry and

colleagues reported that athletes with cognitive and emotional disturbances 1-6 days after an mTBI had significantly reduced NAA/Cr in the dorsolateral prefrontal cortex and primary motor cortex, as well as a depression of Glu/Cr [30]. Vagnozzi and colleagues observed significantly decreased NAA/Cr and NAA/Cho ratios in a group of 40 athletes at 3-, 15-, 22- and 30-days post-concussion, compared to controls, despite recovery of self-reported clinical symptoms between 3-15 days [24]. Similarly, patients who have sustained a mTBI from motor vehicle accidents, falls or assault displayed reduction in NAA/Cr [31, 32] and NAA/Cho ratios [31]. One non-acute study observed increased levels of Cr and Glu + Gln levels following at 13 days which normalised 3- and 5-months post-mTBI [33].

As seen in the abovementioned studies, Cr is commonly used as a denominator to report metabolite quantification, as it is assumed Cr concentration remains unchanged in the diseased brain [34]. However, the changes seen in Cr in some studies after brain injury has called this practice into question [35]. For example, one group has observed increased white matter Cr levels with no changes in white matter NAA after mTBI [36]. As such, some researchers have suggested that caution should be taken when deciding to use absolute concentrations or ratios, to ensure denominator variance does not overpower findings [37].

Whilst current clinical findings suggest that metabolite alterations may assist mTBI diagnosis and patient monitoring, considerable research on the validity of such measures is still needed. For example, there are still numerous differences in methodology (i.e. the use of ratios versus absolute measurements, regions of interest, inclusion criteria, natures of control groups) and larger cohorts and longitudinal studies are needed to gain better insights into the value of ^{1}H-MRS in mTBI.

1.3.2 White matter changes

Diffusion tensor imaging (DTI) is another *in vivo* neuroimaging technique that can be used to observe demyelination, axonal damage, ischemia, oedema and inflammation in white matter by measuring the quantity and direction of water molecule diffusion [38]. DTI parameters include fractional anisotropy (FA) (a measure of anisotropy diffusion), mean diffusivity (MD) (a measure of overall diffusivity), radial diffusivity (RD) (a measure of diffusion perpendicular to axons) and axial diffusivity (diffusion parallel to axonal fibre tracts) [39]. Axonal injury, as a result of acceleration and deceleration forces, is thought to be a major cause of mTBI symptoms [36]. The corpus callosum (CC) is a large white matter tract particularly vulnerable to trauma and which may be responsible for sensory and motor cognition deficits when disrupted and thus is often studied [39]. However, conclusive evidence of DTI sensitivity in the context of mTBI has yet to be found [40], as relatively few studies have investigated acute outcomes after an isolated single mTBI. Some have observed acute white matter changes post-mTBI through increased FA and decreased MD values, potentially due to pathophysiological changes such as axonal cytotoxic oedema and neuroinflammation [41, 42]. In contrast, others have found mTBI resulted in decreased FA along with increased MD, suggestive of reduced white matter fibre density, demyelination, and axonal damage [43-45]. In contrast, one study found no changes in FA and RD values, even though significant cerebral blood flow reduction and neurometabolite alterations were observed [46]. Some have hypothesised that DTI may be a predictive measure of patient outcomes; however, inconsistent findings due to the lack of standardised research and clinical protocols currently limit the application of DTI in mTBI diagnosis [47].

1.3.3 Fluid Biomarkers

Molecules that can be measured or quantified in biological fluid are called fluid biomarkers. Fluid biomarkers serve to identify physiological or pathological processes

occurring in living organisms. In the context of mTBI, fluid biomarkers have considerable appeal, and much research has recently focused on the potential for markers to provide objective and reliable indication of mTBI pathophysiology in order to help diagnose, predict outcomes and optimise management of mTBI. Fluid biomarkers can be collected in numerous sample types, including cerebral spinal fluid (CSF), blood, saliva, urine and tears.

Brain injury can increase the permeability of the blood-brain barrier, allowing increased movement of relevant TBI molecules into the systemic circulation [48]. TBI can also lead to the transportation of potential biomarkers of brain injury through the CSF to the blood via the glymphatic system [49].

Ubiquitin C-terminal Hydrolase (UCH-L1) is a protein almost exclusively found in the cytoplasm of neurons and has previously been found to be acutely elevated in the CSF and serum after pre-clinical and clinical severe TBI [50, 51]. However, the expression of UCH-L1 is not specific to the central nervous system (CNS), as it is also present in the endocrine system, some tumours, endothelial and smooth muscle cells, thus reducing the utility of serum UCH-L1 as a serum biomarker of brain injury [48]. Similarly, neuron specific enolase (NSE) which is also released into the extracellular space after neuronal injury and death, is hypothesised to be an acute predictor of neuronal damage severity, however, NSE expression in other cell types makes it difficult to interpret the specificity of findings [48]. Tau, a microtubule-associated protein (MAP) abundant in axons, but also present in skeletal muscle, the kidneys and testes, has been found to be acutely elevated in plasma relation to mTBI in a number of studies [52, 53]. However, like UCH-L1 and NSE, current assays cannot differentiate between the sources of tau, again posing a limitation on plasma/serum tau as a potential axonal injury biomarker.

The calcium (Ca^{2+}) binding protein, S100B, and glial fibrillary acidic protein (GFAP), are potential biomarkers of astroglial injury, as both are highly abundant in astrocytes [48]. S100B has been quite extensively studied, with elevated serum levels reported during the acute stages of mTBI; however, there is debate as to whether elevation of S100B may not be brain-specific, as S100B can also be released into the blood after muscle injury [54, 55]. Whilst GFAP has been shown to predict abnormalities via CT scan in mTBI patients [56, 57], the specificity and prognostic ability of serum GFAP needs further investigation [48].

Lastly, neurofilament light (NfL), a cytoskeletal protein abundant in most myelinated axons has also been postulated as a potential biomarker of mTBI. Recent studies have indicated that NfL may be a sensitive biomarker of axonal damage neurodegenerative diseases and is thought to be released at higher levels into the CSF [58, 59] and blood [60] following axonal injury or degeneration.

Overall, despite the identification of multiple potential fluid biomarkers of mTBI, issues with factors such as cerebral specificity, poor understanding of temporal profiles due to variable and often single collection time-points, and lack of association with other objective indicators of brain injury and recovery have prevented application of these biomarkers in the clinical setting to date [61].

1.4 Traditional pre-clinical rodent models of mTBI

There is a widely recognised need to increase our understanding of mTBI pathophysiology. However, there are a multitude of confounding factors and limitations when studying mTBI neurobiology in humans. Difficulties with participant recruitment and drop-out are common in mTBI studies, and outcomes can be significantly altered by variability in genetics, pre-existing medical conditions and comorbidities, lifestyle, and

socioeconomic factors. Furthermore, conducting the prospective long-term studies required to increased understanding of the effects of mTBI can be cost prohibitive, and invasive procedures needed to understand the molecular and cellular changes in the brain are not feasible in most mTBI patients.

As such, researchers have developed numerous experimental rodent models of mTBI aimed to mimic the multitude of clinically relevant mechanisms, conditions, and outcomes of injury. Animal models allow researchers to control injury factors, thoroughly investigate behavioural deficits and neuropathological outcomes of TBI, and thus have been essential in furthering our understanding of mTBI in an inexpensive and time efficient manner [11, 62]. Furthermore, pre-clinical experiments allow researchers to evaluate potentially new diagnostic and therapeutic strategies. This review will now briefly outline current animal models of mTBI, highlighting potential strengths and limitations of these models for mimicking clinical mTBI, before describing how these pre-clinical models have increased understanding of neurobiological changes likely to occur in the clinical condition.

1.4.1 Controlled Cortical Impact

Although not exclusive to rodents, the controlled cortical impact (CCI) is a commonly used model of moderate and severe TBI in rats and mice to deliver direct focal damage to the exposed dura [63]. The procedure involves anaesthetising the animal and placing it in a stereotaxic frame before a craniotomy (removal of part of the skull) is performed. A piston is aligned above the exposed dura and driven by a pneumatic or electromagnetic force at a predetermined depth, dwell time and velocity to deliver the injury. To execute a milder CCI, the velocity and depth can be reduced relative to moderate and severe CCI. However, even though some studies have utilised reduced velocity and depth of piston movement in attempt to produce a mTBI, most report extensive structural damage to the cortex, mortality, and severe and/or lasting functional

effects i.e. characteristics more commonly seen in moderate-severe TBI [64-66]. As mTBI is typically a largely diffuse injury without overt focal damage and persisting functional deficits, the clinical relevance to CCI to mTBI is questionable [11].

1.4.2 Fluid percussion injury

The fluid percussion injury (FPI) model aims to induce a mixed focal-diffuse injury to the brain and is among of the most well characterised preclinical models of TBI [67]. Originally designed for larger animals, the FPI model was later adapted for rodent research [68-71]. The model delivers an injury by generating a fluid pulse to a craniotomy located centrally between bregma and lambda on the sagittal suture line (midline FPI) or laterally on the parietal bone (lateral FPI) onto the intact dura of an anaesthetised rodent brain [67, 68]. However, like traditional CCI cortical cavitation can occur, and as will be discussed later in this review, the use of craniotomy and anaesthesia in these models may pose issues in translating neurobehavioural and neuropathophysiological findings to a clinical setting.

1.4.3 Blast Injury

To further understand the injury mechanisms of blast-related TBI in which military service members and veterans are exposed to, laboratories have simulated these unique conditions in a range of ways including, detonating explosive material from a predetermined distance to delivering direct pressure shock waves to the animal [72]. Earlier models induced injury by adjusting the blast level and distance of the blast, researchers can induce mild blast TBIs. One variant of blast mTBI places rodents in an open field, to mimic clinical injury mechanisms. However this model produces varying and complex reflections of shock waves within the confined space [73]. Another recently developed model delivers compressed gas through shock tubes, generating plantar shock waves to produce a more consistent shock wave profile. These models place rodents inside the tube, with the exit closed or open, or place rodents near or further away from

the open shock tube exit to adjust injury severity [73, 74]. Although there are consistent findings that repeated blast mTBI model induce transient hearing loss and ocular injury [75], this technique is relatively new, and more research is required to truly verify whether the contributions of this model are unique from others. Whilst the experimental paradigms in blast injury models more closely reflect those experienced by military personnel [76], some studies have suggested rodent models of blast mTBI may share the same injury mechanisms of other acceleration models [77].

1.4.4 Weight-Drop model

Current weight-drop injury models (e.g. Marmarou's impact) produce a closed-head impact by using a free-falling guided weight onto the exposed skull of an anaesthetised animal, typically resulting in a diffuse brain injury [78-80]. By adjusting the mass and height of the free falling weight, investigators are able to alter injury severity and therefore, and the model has been scaled to study mTBI [78]. Furthermore, this model most often places rodents on a foam platform, to mimic clinical acceleration and deceleration injuries [80]. Although the duration of animal induction in this model is shorter than others, as it omits craniotomy, animals are still at risk of skull fractures on impact, a characteristic rarely seen in clinical mTBI [78].

1.5 Current preclinical mTBI symptomatology

Animal models of mTBI allow for controlled assessment of cognitive, sensorimotor, and behavioural deficits seen in this condition. As mentioned above, due to the heterogenous nature of the injury, there are numerous variations of pre-clinical mTBI models, with each aimed to mimic various clinical mechanisms and as such, behavioural and functional outcomes may vary between models and studies. The onset of clinical mTBI symptoms are often rapid and short lived [2]. However, numerous rodent models of

mTBI may produce injuries that exceed the symptom severity observed in human mTBI. For example, clinical cognitive deficits are a common symptom within the first 24 hours with majority of studies finding resolution within a week post-mTBI [6, 81-83]. This has been replicated in some rodent mild lateral FPI models involving a craniotomy, showing spatial memory deficits at 24 hours that resolved 4 days post-single injury [84]. However, some have observed more persistent deficits. Hylin and colleagues found cognitive deficits present at 5 days after Morris water maze (MWM) [67], whilst another found memory impairment was still present at 21 days post injury using novel object recognition task [85]. Similarly, a study found mice displayed spatial memory deficits in the MWM at 6 days, with spatial learning and memory still impaired at 8-14 days in the Barnes maze after a single closed-head mTBI delivered with a CCI [86].

The onset of motor impairments after a remote mTBI in humans is rapid and thus, is usually assessed acutely in patients [2] with impairments typically resolving within days [87]. However, a number of current pre-clinical models may produce more persistent motor impairments than what is seen clinically. One study in observed balance impairments 3 days post-a closed-head injury delivered using a CCI device, whilst another found a similar injury on anaesthetised mice produced motor deficits on the rotarod that were still present at 7 days post-injury compared to shams [86]. Additionally, another study in rats observed persistent sensorimotor deficits that still remained 28 days after a single lateral FPI in rats [85].

Given that some have speculated that one rat day is equal to approximately 27 human days, as rats have a shorter lifespan with accelerated maturity in comparison to humans [88], the length of persistent symptom outcomes from some rodent studies may be an indication that several current models produce more severe injuries than those seen after clinical mTBI in humans. It is important that rodent mTBI models aim to capture the

severity and transient nature of neurobehavioural disturbances in human mTBI, to confidently capture the neuropathophysiological underpinnings of these symptoms.

1.6 Preclinical neuropathology

As previously mentioned, invasive procedures are not ethically feasible when studying human mTBI, therefore, most understanding of mTBI neuropathology is derived from animal studies. However, as it is widely acknowledged that differences in anatomy, physiology and pathology can be substantial between species, it is important that this understanding is described in the context of animal studies. Although the exact neurobiological mechanisms are still poorly understood, it is widely accepted that mTBI leads to a cascade of neuropathological events that alter the biochemistry of affected neurons and glia, which in turn results in the rapid onset of mTBI symptoms. The insults from TBI are divided into two aetiologies, primary and secondary injury. Primary injury occurs at the moment of impact, often causing immediate damage to neurons, glia, and endothelial cells. Secondary injuries are induced by, and evolve from the primary injuries, and may include mechanisms such as neurometabolic changes, oxidative stress and neuroinflammation [89]. However, much research is still needed to understand these secondary mechanisms and their magnitude following mTBI. Utilising pre-clinical models allows rigorous investigation of the molecular and cellular effects of mTBI to validate potential biomarkers and therapeutic interventions for the injury [11]. As previously outlined, although there are various ways to induce mTBI in the laboratory, the outcomes of these models may share several similarities which indicate that mitochondrial dysfunction, hyper-glycolysis, oxidative stress, axonal damage and inflammation are involved in the neuropathological cascade of mTBI (see Figure. 1.1). A review of studies to reveal these neurobiological changes after mTBI is provided herein.

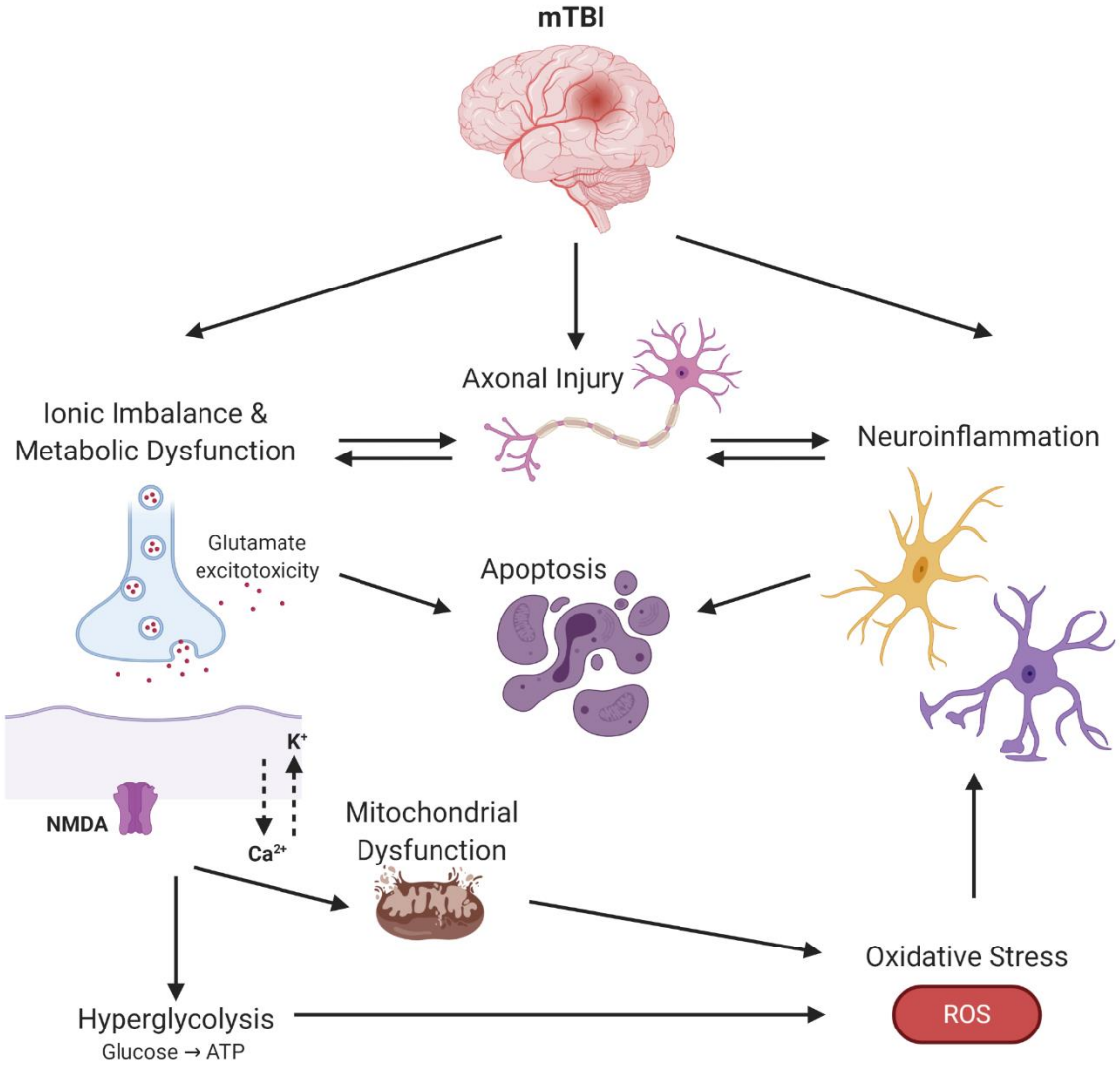

Figure. 1.1 Pathophysiological cascade after mTBI. Created with BioRender.com.

1.6.1 Acute neurometabolic and ionic imbalances

Neurometabolic processes are highly dynamic, and substantial metabolic shifts can occur in an attempt to compensate for physiological or pathological changes. It is well documented that metabolic dysfunction, seen in neurodegenerative diseases, is a major cause of cognitive and neurobehavioural deficits.

In the context of mTBI, biomechanical forces can cause mechanoporation of cell membranes, initiating neurometabolic and biochemical changes, and therefore disruption

to the brain's cellular homeostasis [90, 91]. mTBI immediately leads to uncontrolled depolarisation and excessive release of excitatory neurotransmitters, predominately Glu, causing a buildup of neurotransmitters in the synaptic cleft and can result in Glu excitotoxicity, which can potentially lead to cell death [92, 93]. Additionally, neurotransmitter build up increases the efflux of intracellular K^+ due to Glu binding and activating N-methyl-D-aspartate (NMDA) and D-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors [90]. mTBI can also lead to mechanoporation, allowing non-specific ion fluxes, such as influx of Ca^{2+} resulting in axonal injury [92].

To compensate for the ionic imbalance, ionic pumps requiring adenosine triphosphate (ATP), go into overdrive, leading to depletion of intracellular stores of ATP and an elevation in adenosine diphosphate [90, 94], triggering compensatory hyper-glycolysis (the breakdown of cellular Glc to produce ATP) to increase cellular ATP production [95]. Collectively, these disturbances are often referred to as the 'neurometabolic cascade/crisis' of mTBI, which is hypothesised to be a major contributor to mTBI symptomology.

1.6.2 Mitochondrial respiration

Metabolic and ionic imbalance after mTBI are thought to affect the function of mitochondria [90]. Mitochondria are membrane-bound organelles in charge of energy production, predominantly through the mitochondrial electron transport chain (ETC) [96]. ATP production in the mitochondrial ETC consists of four protein complexes (I-IV) and an ATP-synthase (detailed in Figure. 1.2). However, mitochondrial impairment (induced by Ca^{2+} influx due to TBI) can disrupt the ETC, causing the release of cytochrome C (a proapoptotic protein) and trigger the activation of caspases involved in axonal injury and apoptosis [97-99]. In addition, influx of Ca^{2+} can also activate calpains which are responsible for the breakdown of cell proteins, and have been linked to axonal injury and

degeneration [100]. For example, calpain 10 has been shown disrupt essential components in the mitochondrial ETC thus preventing aerobic respiration [101].

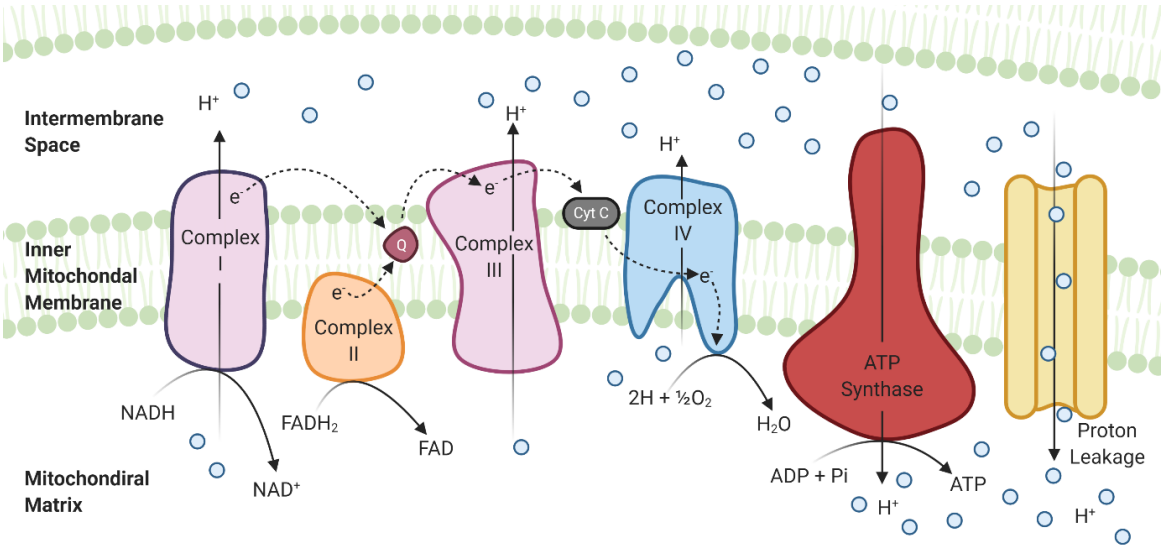

Figure. 1.2. The mitochondrial electron transport chain. The ETC is the main source of ATP production in the mitochondria. Briefly, complex I, II and III are involved in the transfer of electrons through oxidative reactions, resulting in the output of protons (i.e. H^+) in the intermembrane space. Complex IV receives and supercharges these electrons allowing protons to react with oxygen and generate water (H_2O), further adding to proton output into the intermembrane space. This creates a proton gradient which is then used by ATP synthase, allowing the protons to flow from the intermembrane space to the mitochondrial matrix and converting ADP to ATP [102]. However, mitochondrial dysfunction, due to mTBI, can lead to proton leakage, thus disrupting the proton gradient and reducing ATP synthesis. Diagram based on Underwood et al. [103] Created with BioRender.com.

1.6.3 Hyper-glycolysis, mitochondrial dysfunction, and the cellular 'energy crisis'

As previously mentioned, and as recently shown in a single mTBI induced by a weight-drop model in rats [104], mTBI can deplete intracellular ATP [90, 94]. This is thought to trigger two main events to combat depletion: mitochondrial ATP production and hyper-glycolysis. Glycolysis is the breakdown of cellular stores of Glc for ATP, however, is inefficient when compared to mitochondrial ATP production. As such, hyper-

glycolysis is thought to occur, resulting in depletion of Glc and an increase in its biproduct, lactate. Although hyper-glycolysis has been shown to occur in severe TBI [105], to the best of my knowledge, only one rodent study has provided evidence of hyper-glycolysis acutely after an open skull mTBI in rats [94]. However, a more clinically relevant closed-head injury delivered via a weight-drop only found evidence of glycolysis, not hyper-glycolysis, at 48- and 120-hours post-injury [104]. As such, further research, using more clinically relevant rodent models of mTBI, is required to create a temporal profile of Glc metabolism and to determine whether hyper-glycolysis does occur after a remote mTBI.

Mitochondria are essential in regulating cellular metabolism, not only by producing ATP, but also by buffering intracellular Ca^{2+} [106]. However, mitochondrial ability to produce ATP may actually be compromised by mTBI. Supporting this hypothesis, a recent study found significantly decreased expression of mitochondrial ETC proteins involved in the cortex, hippocampus, and cerebellum 7 days after a single mild midline FPI in rats [102]. Mitochondrial impairment may be due to its need to sequester the influx of Ca^{2+} that can occur after brain injury [107]. The demand to buffer the sudden Ca^{2+} influx as well as forcibly producing intracellular ATP can overload mitochondria resulting in mitochondrial dysfunction and leading to oxidative stress [108]. One method to assess mitochondrial function after brain injury is the Seahorse XF Analyzer, which measures the oxygen consumption rate (OCR) of mitochondria in response to inhibitors and substrates of the ETC complexes in real-time [103]. However, few have utilised this technology in rodent mTBI studies. Using the Seahorse FX Analyzer, a single closed-head injury study in mice found a reduction in State 3 respiration (i.e. a measure of oxygen phosphorylation), whilst another found mitochondrial respiration to be altered differently depending on which sub-fields of regions were analysed after a single mild midline FPI [103]. Nonetheless, there is not enough literature to confirm whether

mitochondrial impairment is the key modulator of metabolite alterations observed in clinical studies using the ^{1}H-MRS. As such, more clinically relevant animal research is needed to investigate the neuropathological associations leading to clinical metabolic outcomes.

Additionally, a single mTBI may reduce cerebral blood flow [67] which, paired with the increased energy demand as described, may also result in a disparity in which the energy supplied does not match demand [46, 90]. This mismatch, which may be associated with symptoms of fatigue following mTBI has been termed as a cellular 'energy crisis' [90].

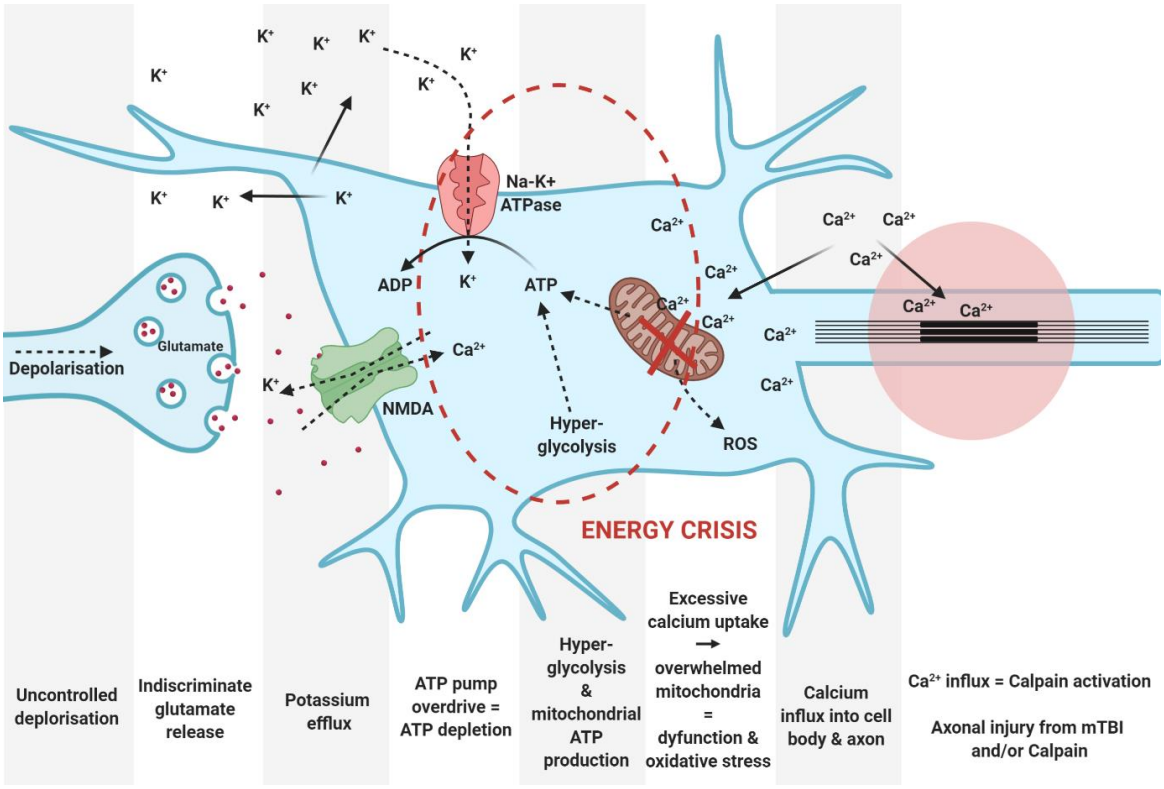

Figure. 1.3. mTBI leads to ionic imbalance and metabolic dysfunction. mTBI can lead to uncontrolled depolarisation resulting in excessive release of Glu into the synaptic cleft. Injury can lead to potassium efflux, further exacerbated by Glu activated NMDA receptors. To restore ionic balance, ATPase pumps go into overdrive, depleting ATP. More ATP is produced by hyper-glycolysis and mitochondria. At the same time, mitochondrial intake of uncontrolled Ca^{2+} influx, overworks and impairs mitochondria, consequently resulting in an energy crisis. Additionally, impaired mitochondria release reactive oxygen species (ROS) leading to oxidative stress. Simultaneously, Ca^{2+} also enters the axon due to axonal shearing, activating Calpain and initiating cellular apoptosis. Axonal injury from mTBI can also destabilise microtubules and compact neurofilaments, interfering with neuronal transmission.

1.6.4 Neuroinflammation

The neuroinflammatory response to trauma is a crucial molecular and cellular component of the CNS [109]. Neuroinflammation involves a multitude of processes, including the activation of microglia and astrocytes which release inflammatory mediators including ROS and inflammatory cytokines, such as interleukin 1 beta (IL-1β),

IL-6 and tumor necrosis factor-α (TNF-α) [109, 110]. Although inflammation is important for clearance of debris and for tissue repair, dysregulated/persistent inflammation can contribute to neurological symptoms and degeneration on [111]. It is well known that neuroinflammation is a key secondary injury mechanism that can have detrimental effects following moderate or severe TBI [112-114], however few studies have investigated the role of neuroinflammatory responses following mTBI.

Neuroinflammation is, however, widely considered to be a key factor in the pathological cascade following mTBI, with a variety of animal models having found evidence of inflammation after a single injury. For example, multiple studies have shown transient increased markers of microglia [84, 115] and/or astrocytic reactivity after a single lateral FPI [84, 115] or closed-head injury by weight-drop [116] at 4-5 days post-impact with resolution at 4-5 weeks. Others have found a single lateral FPI increased markers of microglia and astrocytes within 6 hours after injury that persisted 15-18 days, with no evidence of recovery [112], whilst another found astrogliosis was not present at 1 day, but significantly increased at 3 days that persisted to at least 60 days post-mild CCI craniotomy [117]. However, despite such evidence, it is difficult to reach a consensus of the severity and duration in which neuroinflammation is present after a mTBI in rodents, largely due to the large variety of pre-clinical models used in studies. Importantly, findings from more traditional highly invasive models of mTBI (i.e. involving craniotomy) should be interpreted carefully, as they may produce more persistent and severe inflammatory consequences than those typically seen clinically [11].

Animal models of mTBI should aim to reflect the clinical injury, that is pathophysiological alterations without visible neuronal loss, lesions, contusions or persisting cognitive, behavioural and motor deficits after a single injury. More research is needed to prioritise the use of more relevant models to provide clinically relevant insights

into not only the neuroinflammatory effects, but also the pathological cascade of mTBI, which will better inform potential therapeutic targets after injury.

1.7 Limitations of Animal Models

Animal models have served an important role in developing our understanding of the biomechanical, cellular and molecular characteristics of clinical mTBI [11, 62]. Although these well-characterised models are still widely used to further our understanding of mTBI [118], they may come with some translational limitations.

1.7.1 Craniotomy

Craniotomy allows for precise focal injury and consistency when injuring multiple animals. Traditional injury models such as CCI and FPI require the use of anaesthetics and craniotomy to expose the dura mater for direct impact with little to no movement of the head [119, 120]. However, the procedure is not representative of clinical mTBI (i.e. diffuse injury with absence of skull fracture) with the craniotomy itself shown to affect rat behaviour, induce morphological changes and elevate pro-inflammatory cytokines without a TBI [12].

1.7.2 Head acceleration

Replicating head-acceleration of mTBI in humans is another challenge for preclinical models due to its heterogeneous nature and interspecies disparities. Whilst humans have a gyrencephalic brain, rodents have a smoother brain with less formed sulci [121]. As such, given that gyri can influence brain movement within the skull, rodents may experience much less deformation in response to acceleration, deceleration and/or rotational forces induced by mTBI [121]. Modern weight-drop models already utilise foam platforms to mimic clinical acceleration and deceleration forces after mTBI. However, unmodified traditional CCI, place rodents on solid platforms with head restraint

via ear buds before delivering injury, thus removing the acceleration and deceleration effects of injury. Furthermore, researchers using blast injury models may choose to avoid secondary effects of acceleration injury by restraining the head and/or body of rodents [122], however, this is not a clinically relevant approach.

1.7.3 Anaesthesia

Another confounding factor to these models is the use of anaesthetics during injury. Anaesthetics arguably reduce stress in rodents when performing closed-head procedures, with evidence that stress may induce remodelling of microglia, promoting pro-inflammatory cytokines [123]. However, anaesthesia may compromise pre-clinical mTBI outcomes with some evidence that anaesthetic agents may prevent blood brain barrier disruption [124], inhibit activation of microglia [125], and promoting necrosis of neurons in rats [126]. Additionally, studies suggest that the anaesthetic, isoflurane, may also induce tau phosphorylation [127] as well as cognitive impairment in mice [128]. A systematic review also found anaesthetics to be neuroprotective, likely effecting rodent TBI outcomes and hindering translational ability of potential TBI treatments [129]. Use of anaesthesia also prevents researchers from performing behavioural tests immediately post-injury, and if not allowed adequate recovery time, anaesthetics can hinder cognitive, behavioural, and motor performance, confounding assessments of brain injury.

1.7.4 Injury Severity

As previously mentioned, some studies may exceed severity of injury typically seen in clinical mTBI. For example, due to the lack of consensus in what types of CCI are ‘mild’, findings from studies using this model in this context are variable. Some studies claim to replicate mild injury [64, 65, 130, 131], despite substantially damaging large portions of the cortex. Such persistent pathological alterations and gross anatomical lesions are not characteristics of mTBI and as such, it is imperative to reach a consensus and characterise the consequences of single injuries in rodent models to ensure they

reflect clinical neurobehavioural and neuropathological mTBI outcomes, before implementing the same models for repeated mTBI research.

1.8 New models of mTBI

Confounding factors may be exacerbated when laboratories attempt to mimic repeated mTBI, with repeated exposure to anaesthetics, reopening and closing of sutures and/or craniotomy [132]. Furthermore, more clinically relevant models are needed to better understand the pathophysiological effects following mTBI. As such, more researchers are modifying traditional models or creating new models that more closely reflect clinical mTBI.

A number of laboratories utilise the CCI device to deliver surgery-free closed-head impacts to mice or rats on foam platforms to mimic acceleration, deceleration [133, 134] or rotational acceleration to the unconstrained head, such as the closed-head impact model of engineered rotational acceleration (CHIMERA) [135] in clinical settings. There are numerous surgery-free closed-head weight-drop models featuring breakaway platforms to mimic rotational movements post-impact [136-138]. Additionally, the lateral impact model also mimics clinically relevant rotation and acceleration in rats without craniotomy [139]. However, although these surgery-free models have made vast modifications towards more clinically relevant injuries, they still require anaesthesia to be performed.

The awake “hit and run” TBI model addresses all described limitations by eliminating anaesthesia, surgery and delivering impact to the unrestrained head of mice. This was achieved by suspending anaesthetised mice restrained in a ventilated plastic cone and delivering an impact with a CCI device [49]. Plog and colleagues were able to replicate accelerated motions on impact as well as deceleration injury by building a wooden barrier in which mice struck after impact [49]. Another high throughput model,

by Petraglia and colleagues, utilises a fitted steel helmet secured to retrained awake mice on a foam platform to mimic clinically relevant acceleration and deceleration forces. A modified CCI with a rubber tip impactor is used to deliver the impact delivering a diffuse injury without skull fracture, focal contusion or haemorrhage [140, 141].

Although new rodent mTBI models are essential in providing a more clinically relevant understanding of the injury, rigorous research and collaboration between scientists and clinicians is required to validate new models of mTBI to ensure they reflect clinical injury. Whilst characterising new models is challenging, they will provide more relevant insights into potential translational biomarkers and therapies and help improve medical decisions and ultimately, patient outcomes.

1.9 Repeated mTBI and clinical symptomatology

The majority of remotely acquired mTBI cases appear to resolve without permanent consequences, however, growing evidence indicates that this is often not the case for repeated mTBI exposure. The importance of recovery times has been reported since the 1970's, in which studies found behavioural deficits from brain lesions were less detrimental when serial lesions were spaced by 2 or more weeks [142]. As such, the interval between mTBIs may be important, with growing evidence that repeated injuries in short succession may exacerbate and prolong clinical symptoms and neuropathology. However, despite the increase in understanding of the pathology of repeated mTBI, there is still debate surrounding its associations with persistent and/or progressive neurological diseases, which will be discussed later in this review. As such those constantly exposed to environments that provide opportunity for repeated injury, such as athletes and military personnel may be at risk of developing neurodegenerative diseases, making repeated mTBI a great public health concern.

1.9.1 Increased Cerebral Vulnerability

The aforementioned hypothesis that shorter intervals between repeated mTBIs may lead to poorer outcomes has led researchers to the hypothesis that there may be a time period of increased cerebral vulnerability (ICV) after sustaining a mTBI. Currently, return-to-play or duty for athletes and military personnel after sustaining a mTBI is guided by symptom resolution; however, there is now growing evidence that neuropathological abnormalities may linger even when individuals become asymptomatic post-mTBI [24, 143]. As such, research has focused on understanding the duration and mechanisms of this vulnerability window. For example, studies in both rats and mice have found that mTBI's separated by 1-3 days, but not 5 days or more, resulted in exacerbated and prolonged neurobehavioural disturbances [144, 145], neurometabolic dysfunction [95] and neuroinflammation [145, 146].

Some clinical research suggests recent mTBI may increase the susceptibility for a subsequent mTBI to occur, and if a secondary mTBI does occur, that symptoms may be of greater severity and longer lasting. For example, one group found athletes that sustained successive mTBI's separated by approximately 21 days, potentially showed chronic and exacerbated neurometabolic disruption with ^{1}H-MRS when compared to athletes that sustained a single mTBI only [95]. This is however, one of the only studies in the literature to compare single injury outcomes to repeated injury. Therefore, more research is needed on the temporal effects of single mTBIs to further understand the metabolic state and inflammatory period which leaves the brain in a vulnerable state and sensitive to subsequent brain injuries. Herein, this thesis will now describe the current understanding of the potential short- and long-term neurological consequences of repeated mTBI.

1.9.2 PPCS

As previously mentioned, most cases of mTBI feature a rapid onset of neurobehavioural and physical symptoms that typically resolve within 7-10 days [6]. However, ~15% of individuals with mTBI develop post-concussive symptoms that persist for several weeks or months [2, 5, 6] This is known as PPCS [147]. A history of mTBI is thought to be the strongest predictor of PPCS [7], with the greatest risk potentially when mTBIs occur in short succession i.e. within the period of ICV. However, there is still some doubt as to what extent mTBI history contributes to these symptoms.

Soldiers who had three or more previous concussions reported more post-concussive symptoms for months following their next concussive injury [148]. This was similar to another study which found collegiate athletes with a concussion history of 2 or more, performed significantly worse in reaction time and verbal memory tests after sustaining another concussion than those that sustained a concussion without a previous history [149]. In contrast, a sample from a separate study found that having a history of 1-2 isolated concussions did not result in worsened outcome after a subsequent mTBI compared to those in that had a consecutive concussion in a shorter time frame [150].

However, the etiology of PPCS is still questioned among clinicians as to whether its persistent symptoms have a pathological or psychological basis [151], mainly because the evidence of neurobiological mechanisms of PPCS symptoms are still insufficient. It is generally accepted that the onset of prolonged symptomology (i.e. lasting more than 4 weeks in adults) from a subsequent concussive impact, may be due to a history of brain injury, but may also overlap with personality traits, genetic vulnerability, and pre-existing mental health conditions (e.g. anxiety, depression, traumatic stress etc.) [152, 153]. This can be seen in one study which observed no neurobehavioural or white matter alterations in patients, despite subjects self-reporting chronic post-concussion symptoms [154].

Therefore, it is difficult to know, whether self-reported PPCS symptoms are mimicked by environmental and psychological pre- and post-injury factors.

1.10 Neurological disorders linked to a history of mTBI

Sustaining multiple mTBIs, or more severe TBIs, has been hypothesised to be a risk factor of mental health decline and cognitive impairment and linked to the early development of a number of neurological diseases. Comorbidities make it difficult to directly correlate a single disorder or disease with a history of mTBI. Therefore, more research is required to understand the extent of these neurological disorders and diseases and their association with repeated mTBI.

1.10.1 Depression

In the wake of numerous publicised suicides of professional athletes, the ongoing concerns about the effects of repeated mTBI and mental health decline is understandable [8]. There is some evidence that depression presents more commonly in retired professional footballers with a history of mTBI than retired athletes without a history of head injuries [155]. Furthermore, another study found retired footballers with a lifetime of mTBI displayed more depressive symptoms than their matched controls [156]. Recent studies are now using DTI to investigate the underlying pathological relationships with depression, with one study finding a negative correlation of reduced FA with increased depressive symptoms in retired athletes with mTBI history [157]. This study however used a small sample size, and although challenging, more high-quality longitudinal studies are needed to further understand the correlations between depression with a history of mTBI.

1.10.2 Mild Cognitive Impairment

The diagnostic classification of mild cognitive impairment (MCI), is typically given to individuals later in life, that perform below normal levels of formal neurocognitive testing, but do not meet the diagnostic criteria for dementia [9]. Studies have shown that individuals involved in high contact sport, such as American footballers display a decline in mental health functioning, memory and cognition [9, 158]. However, the associations between MCI and a history of mTBI still remain unclear, largely as the potential cumulative effects of repeated mTBI are difficult to separate from other environmental (e.g. substance abuse) and biological factors (e.g. age) that may contribute to cognitive decline [9]. Additionally, a recently published systematic review found that clinical studies on professional athletes experiencing long-term cognitive deficits varied in their recruitment of subjects, including their definition of concussion, method of assessment for suspected concussion and their adjustment for confounding factors [159].

1.10.3 AD

One of the strongest risk factors of developing late onset AD is moderate to severe TBI [160]; however, it is still unclear whether repetitive mTBI may also increase this risk. Some evidence suggests that dementia may be activated by repetitive head injuries in professional American footballers [9]. One study found after comparing cause of death certificates of 3,439 American football players to the general public, athletes were 4 times more likely to have died with AD and ALS [161], further suggesting that perhaps the environmental nature of the sport (sustaining multiple head injuries) may increase the risk of developing these conditions. However, to gain a more in-depth understanding is needed of how genetic risk factors, (such as being a carrier of the allele of apolipoprotein E (APOE)) and age affect the risk of developing AD and what role repeated mTBIs potentially have on developing this disease by designing carefully planned longitudinal experimental studies using clinically relevant mTBI models.

1.10.4 CTE

In a report dating back to 1928, clinical descriptions of head injury were observed in long-term career boxers. The report described these boxers as having 'punch drunk syndrome' due to their motor and neuropsychiatric symptoms [162]. Although still controversial, this syndrome has been since termed CTE, with symptoms hypothesised to be caused by periodic or repetitive hits to the head transmitting deceleration and acceleration forces to the brain. [8, 141, 162]. These impacts are thought to cause a progressive decline in behavioural, cognitive and/or motor function appearing around 'midlife' or decades after exposure [8, 141, 162]. However, as of 2019, there is still no consensus on the criteria for CTE diagnosis in a living person [163] and as such, the prevalence of CTE is still unknown.

Clinical evidence of the underlying pathophysiological mechanisms of CTE remains limited with many being observational in nature, therefore making it difficult to establish a clinical and scientific consensus of CTE [141]. Furthermore, clinical neuropathological evidence of CTE, to date, has only been gathered from post-mortem studies of those with a history of head injuries. Therefore, researchers and clinicians have been actively collaborating to define the pathology of CTE.

Post-mortem autopsy studies have divided CTE severity into four distinct stages according to macroscopic abnormalities. Characteristic abnormalities are reportedly unusual in early and mild CTE, however, if identified may be revealed in the cavum septum pellucidum and mild enlargement of the temporal and frontal horns of the lateral ventricles [164]. In moderate and severe CTE, gross abnormalities can include overall brain weight decrease [165], atrophy of grey and white matter, CC thinning, ventricle enlargement, cavum septum pellucidum [166] and depigmentation of the locus coeruleus and substantia nigra. Additionally, the phosphorylation of the microtubule-associated protein, tau, is thought to be triggered by repetitive mTBI [167]. The accumulation of

hyperphosphorylated-tau (h-tau) deposits in neurons and astrocytes in the brain, has been established as a unique pathological hallmark of CTE and AD in post-mortem clinical and preclinical studies [8, 77, 141, 162, 164, 168]. However, there is emerging evidence of *in vivo* prognosis of CTE, with a preliminary study on retired American football players finding h-tau accumulation using *in vivo* positron emission tomography (PET) [169].

Due to the relatively recent acceptance of CTE as a neurodegenerative disease, past prevalence of CTE may have been underreported with one study suggesting that although cause of death in former American football players may be labelled as PD, AD and/or ALS, CTE may actually be the primary or secondary cause of death [161]. This is because the pathological abnormalities seen in CTE are also shared with other neurodegenerative diseases [161].

1.11 Exacerbated and prolonged neuropathology after repeated mTBI

There is increasing evidence that repetitive mTBI may correlate with the onset of neurodegenerative diseases [164]. Although neuropathological mechanisms after mTBI have been identified, not much is known about how these processes link to ongoing or potentially progressive pathological deficits after repeated injury. This review will outline the clinical and preclinical findings of known pathological processes correlated to repeated mTBI.

1.12 Cumulative and long-lasting symptomatology after repeated mTBI: Understanding from pre-clinical studies

Due to the varying nature in which human mTBI can occur, preclinical studies vary greatly in how the injury is induced, the severity of injury and total number of impacts. Adding to the challenge of studying repeated mTBI, there is no universal

consensus between human and rodent pathophysiological processes. Interspecies differences between humans and rodents (including but not limited to lifespan, puberty and circadian rhythm disparities), make determining clinically relevant mTBI inter-injury intervals particularly challenging [170]. This adds to the varying intervals of injury between studies and makes translating neurobehavioural and pathological outcomes in repeated mTBI rodent studies particularly difficult. However, despite these challenges, rodents still serve as a useful tool in understanding the behavioural and pathophysiological consequences of repeated mTBI.

As previously mentioned, given that there is no uniform acceleration between rodents and humans, varied inter-injury intervals are used to study repeated mTBI in preclinical studies. However, despite this, there is some consensus as to which injury intervals may potentially occur, given the current return-to-play or duty guidelines following a mTBI. For example, repeated mTBI in both rats and mice with each injury scheduled apart 24 hours may mimic current intervals of 1-2 weeks occasionally observed in sports and military injury settings [11, 88]. As previously mentioned, two mTBI's separated by 1-3 days but not 5 days or more has been found to exacerbate and prolong neurobehavioural disturbances [144, 145]. Notably however, some studies have found injury-intervals greater than 5 days prolong behavioural deficits. For example, Vonder Haar et al. found that 5 CHIMERA injuries in mice, each separated by 14 days, caused progressive impairment in impulsive choice after each injury [171]. Another study found that 5 mTBIs, induced by Marmarou's weight-drop, separated by 24 hours and 1 week, but not 1 month, induced prolonged cognitive deficits [137].

Cumulative injuries have also been shown to exacerbate short- and long-term spatial memory deficits and anxiety-like behaviour as well as cumulative and prolonged depressive-like behaviours [172]. In contrast, some researchers adopt more extreme and frequent injury schedules, with a number of studies inflicting 3 or more injuries a day for

multiple days [140, 141, 173]. However, given the current guidelines preventing individuals returning to play/duty while symptomatic, the injury schedules adopted by these studies may exceed true clinical timelines to repeated injury and perhaps reflect the injury frequency of sub-concussive impacts instead.

Multiple studies have found that repeated injury exacerbates and prolongs neurobehavioural outcomes compared to a single impact in rodents. For example, although injury intervals (24 hours – 5 days) and models vary between studies, repeated mTBI in rodents have been shown to cause cumulative and/or prolonged spatial memory deficits [85, 144, 172, 174-176] when compared to single injury groups. Repeated mTBI [86, 133, 172] also impaired spatial learning compared to single injury. Furthermore, elevated anxiety-like behaviours [133, 172, 174] and depression-like behaviours [172, 174] were observed after repeated mTBI compared to single injury groups. Results have been more varied when assessing motor deficits between repeated and single mTBI rodents, with some finding that repeated injury exacerbated and prolonged deficits compared to single injury in mice [144] whilst others found no differences between single and repeated mTBI rat groups [133, 172].

Finally, the severity of models used in some studies of repeated mTBI may be variable. For example, one study found 4, 5 or 10 impacts separated by 24 hours using a modified weight-drop model did not produce long-term motor, cognitive or spatial learning deficits in mice [136], whilst another found that a single closed-head injury in mice induced by a CCI produced learning deficits that were still evident at 18 months post-injury [132]. As such, characterisation of modified models of rodent mTBI may be imperative to ensure clinical relevance of the injury before continuing to repeated injury.

Overall rodent models have greatly contributed to our current understanding of the effects of repeated mTBI. However, even the seemingly most clinically reflective models

(no surgery or anaesthesia) should include a single injury group to ensure clinical relevance of post-injury effects as well as incorporate study designs that feature clinically relevant injury schedules.

1.12.1 Neuroinflammation after repeated mTBI

1.12.1.1 Clinical findings

As previously mentioned, observing pathological outcomes post-brain injury is difficult in a clinical setting, therefore nearly all clinical investigations of neuropathology after mTBI feature post-mortem analysis of individuals with a remote or recent history of repeated mTBI exposure. For example, one post-mortem study using 66 brains of former American footballers found that although there was no statistical difference in ionised calcium-binding adaptor molecule (Iba-1) and GFAP density (i.e. indicators of microglial and astrocyte reactivity), morphological changes suggestive of glial reactivity such as de-ramification and enlarged cell bodies were apparent in brains of individuals with a history of mTBI. Furthermore, soldiers with a history of two mTBIs and three or more mTBIs had significantly higher CD68 (marker of microglial function) positive cells [177]. Some evidence suggests that h-tau may directly correlate with neuroinflammation. A post-mortem study using brains of former American footballers with or without CTE, found cells expressing AT8 (a marker of tau density) were commonly surrounded by activated Iba1 cells. Furthermore, the study suggests there may be a positive feedback loop between CD68 cell density and AT8 tau pathology, with both positively affecting each other [177]. These findings suggest that chronic neuroinflammation may by associated to years of repeated exposure to sports-related brain injuries.

Although preliminary, a study with retired American football players provided some evidence that using *in vivo* PET h-tau accumulation [169]. PET imaging has also recently been found to successfully detect the increased expression of the translocator protein (TSPO) in activated microglia of former NFL players [178]. Given that microglia

are thought to be persistently activated following repeated mTBIs, PET scans may be a new and promising non-invasive method to observe clinical *in vivo* neuroinflammation.

1.12.1.2 Preclinical findings

Despite variations in preclinical mTBI models and inter-injury intervals, many studies have observed increased and prolonged/chronic microglial activation and astrocytic reactivity following repeated mTBI when compared to single mTBI animals [85, 132, 141, 145, 172, 179, 180].

Other studies have also found increased microgliosis and astrocytic markers after repeated mTBI compared to sham groups [135, 140, 181], however these studies lack single injury groups and therefore, it is unknown whether the injury severity of those models are reflective of pathology specific to repeated impacts. Interestingly, some preclinical models of repeated mTBI have also found increased presence of h-tau and at the same timepoints as increased neuroinflammation [141], providing evidence that mTBI leads to a cascade of interlinking pathological mechanisms, whilst others found no such correlation [132, 136].

1.12.2 Axonal injury and white matter pathology after repeated mTBI

1.12.2.1 Clinical findings

A key feature of mTBI is diffuse axonal injury without gross structural damage, which can be exacerbated following repeated mTBI [144]. As previously mentioned, DTI may be a valuable tool to asses axonal injury in mTBI patients, however no definite trends have been identified in those that experience ongoing symptoms after multiple mTBIs as there is a lack of consensus in the literature as to which parameter (FA, MD, RD and AD) and which brain regions should be considered biomarkers of PPCS [39]. Reduced FA and/or increased MD and/or RD are the most commonly reported DTI findings in patients with a history of repeated mTBI, with evidence suggesting that may

these measures are more pronounced in patients with PPCS [46, 182-184]. These changes were not dependent on the type of mTBI sustained by patients (blast, sports-related concussion, assault). However, DTI parameters were unchanged in all studies, with one finding no alterations of FA, MD or RD in the white matter tracts of patients with chronic post-concussive symptoms [154]. As advanced DTI is a relatively new measure for mTBI pathology, more research is required to determine specified biomarkers for PPCS as well as track longitudinal changes in DTI parameters.

1.12.2.2 Preclinical findings

Unlike clinical studies, which are subject to participant availability or must wait till post-mortem analysis, rodent models of repeated mTBI allow researchers to investigate the injury profile of axonal injury and diffuse injury at varying timepoints. One technique to investigate the presence of axonal injury may be found through the traumatic axonal injury marker, beta-amyloid precursor protein (APP) immunoreactivity. Studies show elevated APP levels in mice with injuries separated by 24 hours compared with a single mTBI [185] whilst another found subtle APP upregulation in single and 2x mTBI rats using the same inter-injury interval [180]. Longhi et al. demonstrated that a subsequent mTBI at a 3-d inter-injury interval, exacerbated axonal damage in mice [144] whilst another found exacerbated and prolonged microglial activation and axonal damage in the same regions of interest after 2 closed-head injuries delivered by a CCI compared to a single injury [180]. A long-term study conducted by Mouzon and colleagues also observed exacerbated profiles of APP in 2x mTBI animals compared to single mTBI at 6- and 12-months post-injury [132]. Furthermore, the presence of axonal injury is often associated with cognitive, behavioural, and motor deficits [174, 180, 181]. However, this is not demonstrated in all studies, with one finding no evidence of axonal injury despite cognitive deficits after repeated injury at short inter-injury intervals using Marmarou's weight-drop model [137].

As mentioned above, DTI parameters can also inform clinicians and researchers of microstructural alterations in white matter and therefore axonal injury [39]. Few studies have investigated DTI parameter changes after repeated mTBI in rodent models, however some preclinical findings have reflected clinical discoveries, including reduction of FA values in the CC [186]. Others have found increased microglial activation and APP positive markers in the CC alongside significant decreases in MD and AD in this brain structure [187]. These findings further highlight the interconnecting cascade of pathological events that occur after mTBI and repeated mTBI, and more research is required to understand these relationships.

1.12.3 Neurometabolic dysfunction after repeated mTBI

1.12.3.1 Clinical findings

As previously stated, the forces from a mTBI may cause acute and transient metabolic and ionic disturbances. However, sustaining multiple mTBIs may exacerbate and prolong these alterations. Supporting this hypothesis, a pilot study using the ^{1}H-MRS found athletes experiencing a second mTBI, within 15 days of the first injury, had exacerbated brain NAA/Cr reductions at 15 days, and prolonged neurometabolic alterations to 30 days, when compared to singly concussed athletes and control groups [188]. In addition, Johnson et al. found significant decreases in NAA/Cho and NAA/Cr ratios following mTBI, as well as prolonged metabolic alterations when patients experienced multiple concussions [143]. However, some evidence suggests that using absolute concentration levels of a single neurometabolite is a more accurate measure to quantify change and that the assumption that Cr levels are stable throughout the brain may confound results [35]. Finally, Iraq war veterans exposed to repeated blast injuries displayed long-lasting (3.5-year average after final injury) decreased cerebral metabolic rate of glucose compared to control subjects, suggesting the presence of hypometabolism [189]. Mitochondrial dysfunction is hypothesised to be a key contributor to metabolic

impairment observed after repeated mTBI, however more research is needed to understand the specific underlying mechanisms for neurometabolite alterations observed in the ^{1}H-MRS. Utilising clinically relevant rodent models of mTBI can help improve our current understanding of these changes.

1.12.3.2 Preclinical findings

Like all preclinical to clinical studies, there are some practical limitations when translating rodent ^{1}H-MRS techniques to humans. For example, homogenous tissue sampling can be difficult to translate due to interspecies differences, voxel size and positioning disparities. However, recent advances such as improved coils and higher field strengths have increased the sensitivity and spectral resolution of ^{1}H-MRS in rodents, and as such, rodents can help researchers further explore the possibility of ^{1}H-MRS as a biomarker for repeated mTBI.

Some studies have investigated neurometabolite changes after repeated mTBI in rodents. One group found alterations in numerous mitochondrial-related metabolites (including ATP and ADP) following two mTBIs separated by 3 days in rats using ^{1}H-MRS revealed a decreased concentration of numerous metabolites including NAA and ATP but not in injuries separated by 5 days [95]. However, 2 mTBIs separated by 3 days was found to decrease Glc utilisation, potentially indicating hypometabolism [145]. Interestingly, another study found that a single and consequent injury (separated by 20 days) resulted in a transient increase cerebral Glc utilisation providing preliminary evidence that the period ICV may be quite extensive. Overall, all mentioned studies highlight the importance of injury intervals between mTBI and provide evidence of the period of ICV.

Very few studies have investigated the effects of repeated mTBI on the mitochondrial respiration. Using the Seahorse XF Analyser, Hubbard and colleagues

(2019) found a single mTBI reduced State 3 OCR compared to shams, whilst two mild closed-skull delivered using a CCI in rats with an inter-injury interval of 2 days but not 3 days, prolonged lowered State 3 OCR, using the Seahorse XFe24 Flux Analyser [190]. Interestingly this difference was observed at 3 days post- but not 2- days post-final injury [190]. However, this study utilised anaesthesia with an incision site before delivering impacts with a CCI model. As such, more research is required to determine the mitochondrial profile of both single and repeated mTBI in more clinically relevant models of injury, using the Seahorse XF Analyser. Moreover, further research is required to understand whether mitochondrial dysfunction after mTBI can be indirectly assessed using neuroimaging modalities such as ^{1}H-MRS.

1.13 Summary and Aims

The cognitive, behavioural, and neuropathological consequences of mTBI and repeated mTBI are vast, and still poorly understood. Due to difficulties in studying humans, it is imperative that rodent mTBI models replicate clinical injury to gain further insights into neuropathology, so as to facilitate discovery of potential biomarkers and treatment strategies that may improve mTBI management. However, given the confounds of several other rodent models of mTBI, alternative models may be better suited to provide such insights. Accordingly, in this PhD thesis I have co-developed a novel awake closed-head injury (ACHI) model in rats which avoids the use of confounding anaesthesia and surgery, features clinically relevant head acceleration, and allows for immediate behavioural testing post-impact. To ensure the ACHI model produced a clinically reflective mTBI, the aim of **Chapter 2** was to characterise the neurobehavioural and neuropathological effects of a single ACHI.

There is still controversy and debate on the associations between repeated mTBIs and progressive neurological conditions and as such, requires more research. By using a clinically reflective injury schedule and model of mTBI, as well as next generation techniques for tissue analysis, this work added new valuable insights into more translatable pathology and biomarkers to improve clinical management of injury. As such, **Chapter 3** aimed to determine the acute and chronic behavioural changes, blood and neuroimaging biomarkers of repeated ACHIs separated by a 48-hours inter-injury schedule. Finally, metabolic alterations have long been hypothesised to be a major mechanism of the neuropathological cascade after mTBI and can be further exacerbated and prolonged after repeated injury. Whilst some clinical studies have postulated that the advanced neuroimaging technique, ^{1}H-MRS, may reveal potential metabolic biomarkers of mTBI, more pre-clinical studies are needed to reveal the underpinning neuropathology leading to detected metabolite alterations post-single and repeated mTBI. Therefore, **Chapter 4** aimed to investigate the efficacy of ^{1}H-MRS and the underlying pathological changes after single and repeated ACHI.

Chapter 2: Mild closed-head injury in conscious rats causes transient neurobehavioural and glial disturbances: A novel experimental model of concussion

The information presented in this chapter has been published prior to the completion of this thesis. The full PDF copy of this manuscript is attached in **Appendix D**. Author contribution state can be found in **Appendix B**. Copyright permissions can be found in **Appendix C**.

Journal article: **Pham, L.,** Shultz, S. R., Kim, H. A., Brady, R. D., Wortman, R. C., Genders, S. G., Hale, M. W., O'Shea, R. D., Djouma, E., van den Buuse, M., Church, J. E., Christie, B. R., Drummond, G. R., Sobey, C. G., & McDonald S. J., (2019). Mild closed-head injury in conscious rats causes transient neurobehavioral and glial disturbances: a novel experimental model of concussion. *Journal of Neurotrauma*, *36*(14), 2260-2271.

2.1 Introduction

Brain concussion, a common subset of mTBI, is a serious medical and societal issue [2] In the United States alone the incidence of concussion is reported to be 150,000 cases per year [191], with evidence of gross underreporting indicating that the true prevalence is much higher [4]. Concussion is particularly common in sports with the highest participation rates for adolescents and young adults [192], and is increasingly evident in military personnel following war zone blast exposure [193].

Although the neurobehavioural (e.g. cognitive and emotional impairments) and physical (e.g. headache, sensorimotor deficits) effects of concussion typically resolve within 7-10 days, 10-15% of individuals develop post-concussive symptoms that persist for several weeks or months [2, 5, 6]. Despite recent advances in neuroimaging and fluid biomarkers of concussion, our understanding of the specific neurobiological mechanisms underlying the clinical features of concussion and post-concussion syndrome is lacking. In addition, although there is some evidence that exposure to multiple concussive impacts may lead to lingering or even chronic impairments, including neurodegenerative diseases such as CTE, the potential cumulative and chronic effects of repeated concussions are intensely debated and poorly understood [194]. In particular, the existence of CTE as a distinct neuropathology induced by repeated head traumas remains controversial, and

factors such as the number, timing and severity of injuries that may lead to the development of CTE are unknown [194-196].

Given the inherent difficulties in studying the mechanisms and effects of concussion in humans, it is not surprising that much of our understanding is derived from pre-clinical models. Variants of FPI, CCI and weight-drop injury have provided insights into the potential neurobehavioural and neurobiological consequences of single and repeated mTBIs [1, 197, 198]. Nonetheless, potential limitations to the relevance of the aforementioned models to clinical concussion has been increasingly acknowledged in the field. There are various potentially confounding effects on both brain pathology and animal behaviour due to volatile anaesthetics [199-206], surgical procedures including craniotomy [12, 207], re-opening of sutured wounds for repeated injuries, and analgesics [203]. In addition, although several rodent studies have demonstrated cumulative effects of repeated mild head traumas [11, 146], the severity of injury may exceed that of a truly concussive impact with respect to structural damage or persistent neurobehavioural deficits from a single injury, or else may lack any clear single injury outcomes.

To further our understanding of the neurobiological basis and effects of concussions, it is desirable that pre-clinical studies utilise injury models that closely mimic a single concussive incident in humans. Therefore, I have further modified the recently developed awake closed-head injury (ACHI) model in rats [208], and aimed to characterise neurobehavioural consequences of a single ACHI. In addition to eliminating the potential confounds of anaesthetics and analgesics, the priority for this high-throughput model was to ensure that it resulted in the rapid onset of a range of short-lived neurobehavioural symptoms typically seen in clinical concussion. Importantly, the omission of surgery and anaesthetic allowed for acute neurobehavioural testing (i.e. from 1 minute post-ACHI), akin to sideline assessments in sports-related concussion [209].

Although some recent studies have incorporated acute neurological severity scale assessments shortly after impact [208, 210], to our knowledge this is the first study to conduct a sequence of detailed, traditional neurobehavioural tasks on rodents within the first 20 minutes of injury. Finally, given the potential role of a neuroinflammatory response in concussion symptomology [211], I aimed to create a temporal profile of glial reactivity following a single ACHI.

2.2 Materials and Methods

2.2.1 Animals

Male Long Evans rats (n=116) were obtained at post-natal day (PND) 28 from Animal Resource Centre (WA, Australia). Upon arrival, rats were housed in groups of two at the La Trobe University Central Animal House, with a 12 hours:12 hours light/dark cycle and food and water available *ad libitum*. All procedures were approved by the Animal Ethics Committee at La Trobe University (AEC 17-06), and were within the guidelines of the Australian code of practice for the care and use of animals for scientific purposes by the Australian National Health and Medical Research Council (NHMRC). Upon arrival, all animals were given 48 hours to acclimatise to their enclosures before experimenter contact. After this period, animals were handled on four days per week until sham or injury procedures on PND 46.

2.2.2 Restraint Acclimatisation

A disposable DecapiCone (Braintree Scientific Inc., MA, USA) was used to restrain each rat during sham/ACHI procedures (Figure. 1A) [208]. These clear plastic cones feature an opening for the snout to provide ventilation. Animals were led into the restraint cone headfirst and briefly immobilised by holding the entry point of the cone

closed behind the haunches. Rats were acclimatised to the restraint cone for three consecutive days prior to sham/injury procedures. Twenty-four hours prior and at the time of sham/injury, as previously described [208], rats were given a score of 1-4 based on their willingness to enter the restraint cone, as well as movement and vocalisation during restraint. Following restraint acclimatisation, animals were randomly assigned into sham or ACHI groups. There were no differences in restraint scores between groups.

2.2.3 Awake Closed-Head Injury

Our model of concussive-like injury (Figure. 2.1A) was modified from a recently developed ACHI model used with PND 25-28 rats [208] (rationale for modifications detailed in **Appendix A**). In our model variant, un-anaesthetised PND 46 rats were immobilised using a restraint cone and carefully fitted with a 3-Dimentional (3D) printed steel helmet (Figure. 1B), such that the target impact site was positioned over the left parietal bone. An elastic band was strapped under the head to secure the helmet in place during injury. Subjects were carefully lowered onto a foam platform (3" thick Super-Cushioning Polyurethane Foam Sheet, McMaster-Carr, OH, USA) so that the helmet target was directly underneath a steel impactor tip. A CCI (Leica Biosystems, IL, USA) device was connected to a stereotaxic frame and an electromagnetic piston to drive the impactor tip (velocity of 6.5 m/s; extension depth of 10 mm; dwell time of 100 ms) at a 14° angle onto the helmet target. The extension depth was matched by the buffering capacity of the foam platform, and the 14° angle ensured that the impact tip was perpendicular to the helmet surface. Sham procedures were identical to those for the ACHI, except that the impact was triggered adjacent to the animal's head. The total duration of restraint during each procedure was less than 1 min. Animals were removed from the restraint cone immediately post-impact for neurobehavioural assessments.

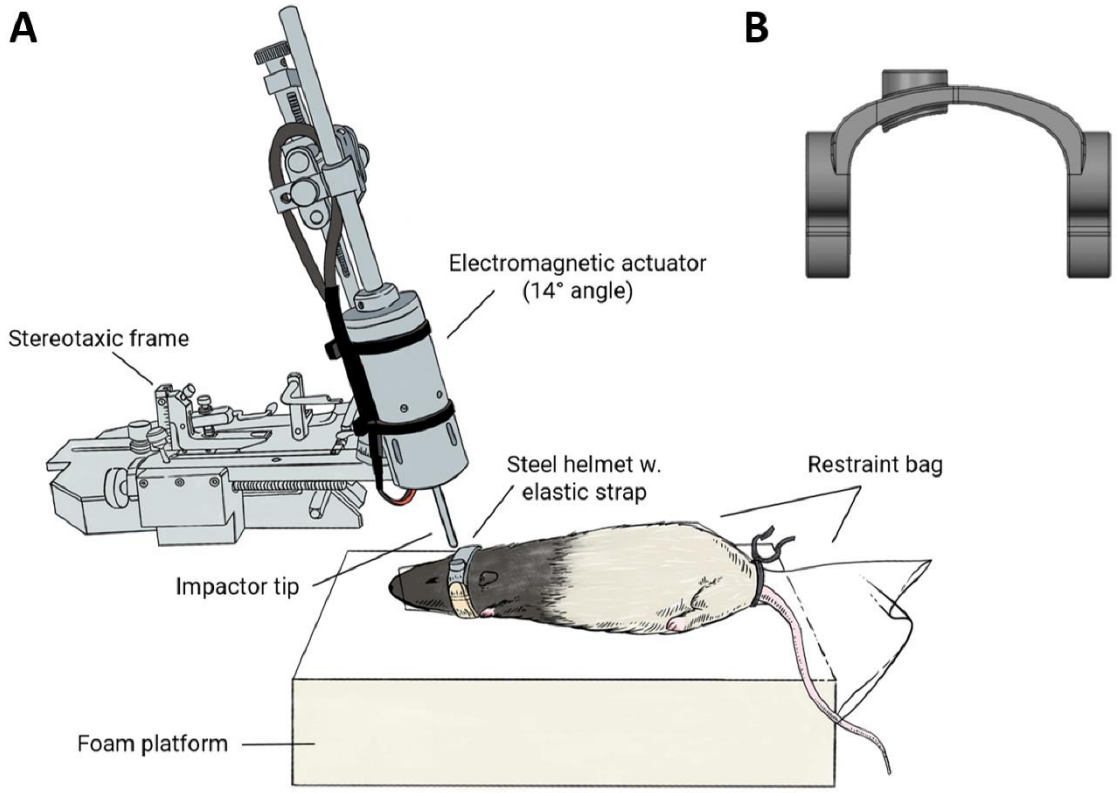

Figure. 2.1. The ACHI model in rats. **(A)** A CCI device was attached to a stereotactic frame, allowing the impactor tip to be positioned at a 14° angle. The rat is placed in a cone-shaped restraint bag and fitted to the 3D printed steel helmet **(B).** The helmet is secured to the head with an elastic band so that the impact target is centred over the left parietal bone. The restrained rat was placed on a foam platform and the impactor tip is centred to strike the helmet target to deliver an injury. The procedure can be completed in less than 1 min. Diagram drafted by Louise Pham, recreated by Giles Watson.

2.2.4 Neurobehavioural Testing

Rats underwent neurobehavioural testing at <20 min, 24- and/or 48-h, and were assigned into 4 groups (Table. 2.1) to avoid the potential confound of repeat testing in the open field (OF), elevated plus maze (EPM) and Y-maze. All behavioural assessments, excluding beam walk, were recorded by an overhead camera and analysed using tracking software Ethovision (Ethovison XT 10, Noldus, The Netherlands). In an attempt to

control for any effects of restraint, rats in the 24- and 48-hours recovery groups were briefly restrained at the same time interval prior to each testing task as performed for the acute injury group. All tasks were performed with paired sham and ACHI rats, with experimenters blinded to the rat groups.

Table. 2.1. Neurobehavioural Groups

	Group A ***(n=15-16/group)***	***Group B*** ***(n=9-10/group)***	***Group C*** ***(n=9-10/group)***	***Group D*** ***(n=17-18/group)***
<20 min	Beam, OF, EPM	Y-maze		
24 h	Beam	OF & EPM	Y-maze	
48 h				Y-maze

OF, open field; EPM, elevated plus maze

2.2.5 Beam Walk

Fine motor function was assessed at baseline, 1 minute and 24 hours post-injury in a similar manner to that described previously[212]. Briefly, the apparatus consisted of a 2 cm-wide, 1.5 m-long wooden beam elevated 75 cm above ground, with a thin mattress placed below to soften any falls. A dark 'home box' was placed at the end of the beam with home cage bedding inside to attract rats to complete the task. At the other end of the beam was a bright light (i.e. an aversive stimulus), as well as a camera to record the task. To complete each trial, rats were required to traverse 1.0 m to the home box. Beam training took place 3 days prior to sham/ACHI and required subjects to cross a 4 cm-wide beam for 5 consecutive trials, followed by a further 5 trials on a 2 cm-wide beam. Rats were gently guided across the beam during training until they each readily crossed without interference of the experimenter. For beam testing, rats were required to traverse the 2 cm-wide beam for 5 consecutive trials before being returned to home cages. Trials began and finished when the nose of the rat crossed the start and finish line, respectively.

Baseline tests were carried out on the two consecutive days prior to injury, with results from both days averaged to produce baseline values for each rat. In a small number of cases, trials were re-performed if rats were immobile for longer than 5 sec. Average latency to cross the beam and total number of hind-limb slips were later determined through captured video analysis by a researcher blinded to the experimental conditions.

2.2.6 Y-maze

Spatial memory was assessed at either 5 min, 24- or 48-hours post-sham/ACHI using the Y-maze similar to that described previously [213, 214]. Briefly, rats were introduced into a Y-maze for a 10-minutes exploration phase, with the novel arm closed, then returned to their home cage. One hour later, rats were reintroduced to the Y-maze for a 5-minutes testing phase with all arms open. For rats in the 5 minutes post-sham/ACHI assessment group, the exploration phase was conducted exactly 55 minutes prior to injury i.e. 1 hour prior to testing as for the 24- and 48-hours assessment groups. Total time spent in the novel arm, the familiar arm (expressed as mean of two familiar arms), and total distance travelled were determined.

2.2.7 OF

An OF was used to assess locomotion/activity at 10 minutes and 24 hours post-sham/ACHI similar to that described previously [215, 216]. This task consisted of a 100 x 100 cm sawdust-covered arena, with 40 cm-high walls to prevent escape. Rats were released into the centre of the field and allowed to explore for 5 minutes before they were returned to their home cage. Total distance travelled was determined.

2.2.8 Elevated Plus Maze

Anxiety-like behaviour and activity were assessed using an EPM similar to that described previously [217-220]. The maze is characterised by a plus-shaped platform

elevated 50 cm above ground. It consists of two open and two closed arms (50 × 10 cm/arm) and a square (10 x 10 cm) central platform. Rats were placed into the centre of the EPM at 15 minutes or 24 hours post-sham/ACHI, and allowed to explore the arms of the maze for 5 minutes before returning to their home cage. Duration spent in the starting centre zone, open-, and closed- arms, as well as distance travelled were quantified.

2.2.9 Brain Collection

Rats were anaesthetised with Lethabarb® and transcardially perfused using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before brains were removed and post-fixed with PFA at 4°C overnight. Samples were transferred into 30% sucrose solution for 4-5 days before being frozen using 2-methylbutane and dry ice and stored at -80°C.

2.2.10 Nissl Staining

Nissl (cresyl violet) staining was used to observe morphological changes of brains collected at 1-, 3- and 14-d post-sham/ACHI (n=3 sham, n=3 ACHI/time-point. Thirty µm coronal sections were collected at the centre of the intended impact site (bregma -4.0 mm) and mounted on slides for staining. Frozen sections were washed briefly in water before they were briefly immersed in 100% ethanol. Sections were then submerged in 100% xylene for 15 minutes to de-fat the tissue, before they were re-immersed in 100% ethanol and then rehydrated with water. Sections were stained in 0.1% cresyl fast violet for 5 minutes and washed in water before they were cover-slipped with DPX Mountant (Sigma, NSW, Australia). Entire sections were taken using a Leica MZ6 stereo microscope to observe any overt structural changes. Images of the cortex were captured at 100x magnification using an Olympus fluorescence microscope to determine the presence of dark stained cells.

2.2.11 Immunofluorescence

Immunofluorescence staining was completed on 35 rat brains collected at 1-, 3- and 14-d post-injury (n=5-7/group at each time-point) to evaluate microglial and astrocytic density after a single ACHI. Coronal sections (14 µm) were collected at bregmata -3.5, -4.5 and -5.5 mm for immunofluorescence. Frozen sections were thawed and incubated in 4% PFA for 5 minutes before they were rehydrated with 0.01M PBS. Sections were then blocked in 10% normal goat serum (Sigma, NSW, Australia) in PBS containing 0.2% Triton-X-100 for 1 h. Sections were incubated at 4°C overnight in primary antibodies: Iba-1 (Rabbit anti-Iba-1, 1:1000, Wako USA Inc, VA, USA) or GFAP (Rabbit anti-GFAP, 1:500, Abcam, Cambridge, United Kingdom). Secondary antibodies for Iba-1 (Alexa Fluor™ 594 goat anti-rabbit, 1:500; Invitrogen, CA, USA) and GFAP (Alexa Fluor™ 488 goat anti-rabbit, 1:300; Invitrogen, CA, USA) were applied to sections the following day for 2 hours at room temperature. Sections were washed with PBS and mounted using Vectashield® Mounting Medium for fluorescence containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., CA, USA), and a coverslip applied before imaging. Captured images were centred on the ipsilateral or contralateral parietal cortex (PCX), CC and dentate gyrus (DG) at 200x magnification using an Olympus fluorescence microscope. Percentage area above threshold for Iba-1 and GFAP was analysed using ImageJ version 1.52e for Windows (National Institutes of Health, MD, USA) by a researcher blinded to the experimental conditions. Images were converted to 8-bit, and a custom threshold was chosen for each batch of staining (with paired sham and ACHI samples per time-point) to control for any inter-batch staining variation.

2.2.12 Statistical Analysis

All outcomes were analysed using GraphPad Prism version 7.04 for Windows (GraphPad Software, CA, USA). All behavioural and immunofluorescence data was analysed with two-way ANOVA and is presented as mean + SEM. Y-maze time in novel arm was also analysed using unpaired student t-test at each timepoint. Two-way repeated measures ANOVA was used for beam task analysis only. Holm-Sidak's multiple comparisons were performed as appropriate. Statistical significance was accepted if $p<0.05$.

2.3 Results

2.3.1 Sensorimotor deficits following ACHI

Sensorimotor function was assessed using the beam task at baseline, 1 minutes and 24 hours post-injury (Figure. 2.2). Total number of hind-limb slips (Figure. 2A) were analysed by two-way repeated measures ANOVA, revealing a main effect of injury ($F_{(1, 29)} = 16.83$, $p<0.001$) and time ($F_{(2, 58)} = 21.22$, $p<0.0001$), and a significant interaction of injury by time ($F_{(2, 58)} = 15.49$, $p<0.0001$). Post-hoc analysis found ACHI rats displayed significantly more hind-limb slips at 1 minutes post-injury compared to sham rats (Figure. 2.2A, $p<0.0001$), with total number of slips resolving back to sham levels after 24 h. Notably, all 15 ACHI rats had an increased number of hind-limb slips immediately post-impact when compared to their baseline performance. There were no differences between ACHI rats and sham rats in the time taken to traverse the beam at all time-points (Figure. 2.2B).

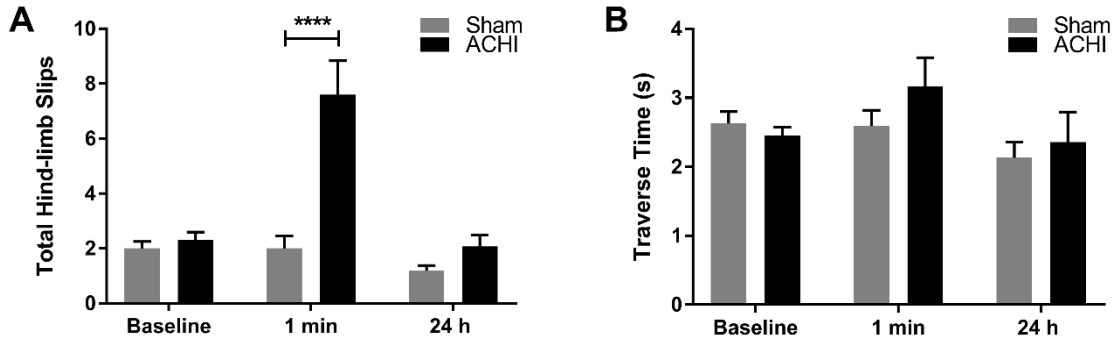

Figure. 2.2. The effect of ACHI on beam task performance. **(A)** At 1-minute post-ACHI, rats displayed more hind-limb slips compared to sham rats (****$p<0.0001$), with resolution back to baseline after 24 h. **(B)** Despite the difference in foot slips at the 1-minute time-point, there were no differences in the time needed to traverse the beam at any time-points. n=15-16/group at each time-point (repeated testing). Data presented as mean + SEM.

2.3.2 Impaired spatial memory post-ACHI

The effect of an ACHI on spatial memory of the Y-maze was tested at 5 min, 24 hours and 48 hours post-injury in different cohorts of rats per time-point (Figure. 2.3A-C). At 5 minutes post-sham/ACHI, two-way ANOVA for time spent in the novel- compared to familiar-arm (Figure. 2.3A) demonstrated a main effect of arm preference ($F_{(1, 34)} = 7.82$, $p<0.01$), with a significant interaction of injury by arm preference ($F_{(1, 34)} = 13.12$, $p<0.001$). Multiple comparisons revealed that at 5 min, sham rats had significant preference for the novel- over the familiar-arm when compared to ACHI rats ($p<0.001$). For preference of the novel arm compared to the familiar arm at 24 hours post-sham/ACHI (Figure. 2.3B), two-way ANOVA revealed a significant main effect of arm preference ($F_{(1, 34)} = 9.55$, $p<0.01$) and interaction of injury by arm preference ($F_{(1, 34)} = 9.53$, $p<0.01$). Post-hoc comparisons found that at 24h, sham rats had significant preference for the novel- over the familiar-arm compared to ACHI rats ($p<0.001$). At 48

h, a main effect of arm preference was found (Figure. 2.3C, $F_{(1, 66)} = 22.91$, $p<0.0001$), with no interaction of injury by arm preference. Post-hoc analysis of 48 hours results revealed a significant preference for the novel arm over the familiar arm in both sham ($p<0.001$) and ACHI rats ($p<0.01$). No differences in distance travelled were found in sham and ACHI rats across all time-points (Figure. 2.3D).

Further analysis was conducted using a two-way ANOVA with time spent in novel arm only, and found a main effect of injury ($F_{(1, 65)}=16.17$, $p<0.0002$) without a main effect of time ($F_{(2\ 65)} = 0.331$, $p<0.72$) and no interaction of time and injury ($F_{(2\ 65)}=2.736$, $p<0.072$). Post-hoc analysis found injured rats spent significantly less time in the novel arm at 5 min ($p=0.003$) and 24 h ($p=0.039$) but not at 48 h ($p=042$) post-ACHI compared to sham rats. Unpaired t-tests (corrected for multiple comparisons, alpha=0.17) were also run for each timepoint for time in novel arm only and found ACHI rats spent significantly less time in the novel arm at 5 min ($p=0.006$), 24h ($p=0.014$) but not 48h ($p=0.43$).

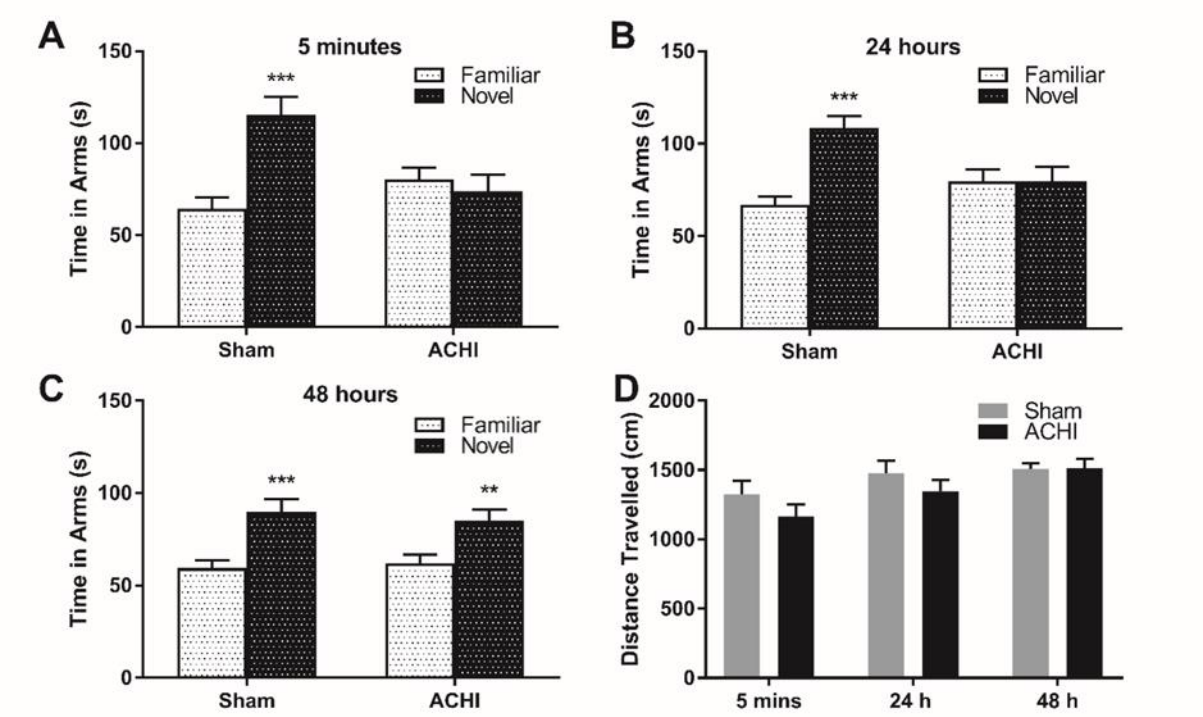

Figure. 2.3. Y-maze performance following ACHI. **(A)** Sham rats spent more time in the Y-maze novel arm than familiar arm when compared to ACHI rats at both 5 minutes (***p<0.001) and **(B)** 24 hours (***p<0.001) post-sham/ACHI. **(C)** At 48 hours, both sham (***p<0.001) and ACHI (**p<0.01) groups spent more time in the novel arm compared to familiar arm. **(D)** There were no differences in distance travelled in the Y-maze between at all-time points. n=9-18 per group at each time-point (no repeated testing). Data presented as mean + SEM.

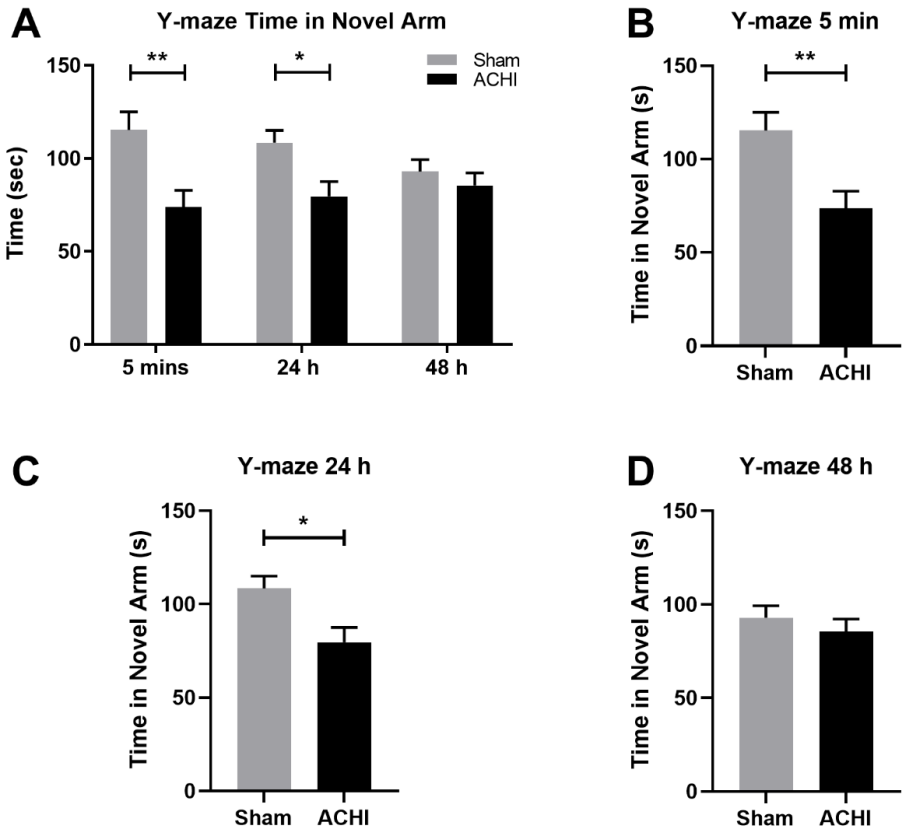

Figure. 2.4. Y-maze time in novel arm following ACHI. **(A)** ACHI rats spent less time in the Y-maze novel arm when compared to sham rats at 5 minutes (**p=0.003), 24 hours (*p=0.039) but not at 48 hours (p=0.418) post-sham/ACHI. Using unpaired t-tests (alpha=0.017) found ACHI rats spent less time in the novel arm compared to sham rats at **(B)** 5 minutes (**p=0.006), **(C)** 24 hours (*p=0.014) but not at **(D)** 48 hours (p=0.425) post-sham/ACHI. n=9-18 per group at each timepoint (no repeated testing). Data presented as mean +SEM.

2.3.3 No differences in OF

The OF was used to observe the locomotion, activity and anxiety-like behaviour of rats at 10 minutes and 24 hours post-sham/ACHI (Figure. 2.5), with no significant differences found between groups.

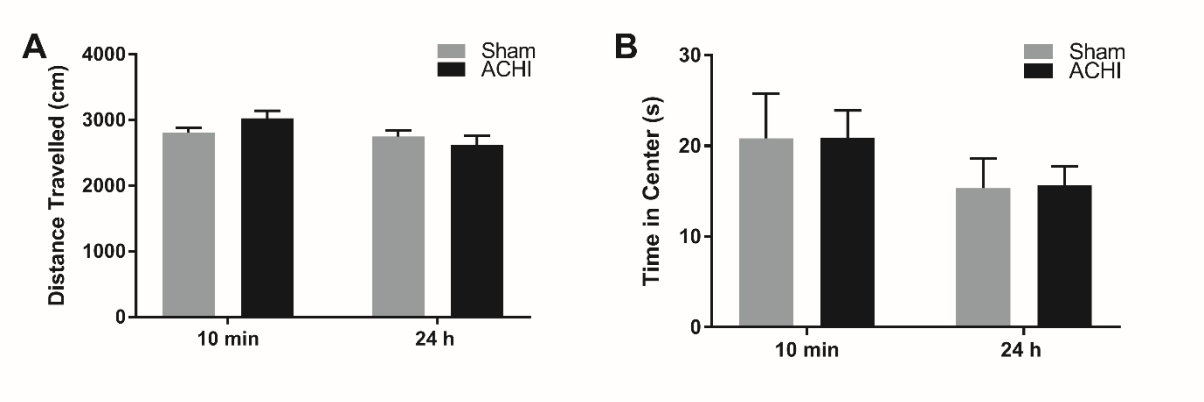

Figure. 2.5. Performance in an open field. There were no differences between sham and ACHI rats in distance travelled at both 10 minutes and 24 hours post-sham/ACHI. n=9-16 per group at each time-point (no repeated testing). Data presented as mean + SEM.

2.3.4 Reduced exploratory behaviour in EPM

Anxiety-like and exploratory behaviour were tested using the EPM at 20 minutes and 24 hours after sham/ACHI (Figure. 2.6). For the duration spent in the EPM closed arm at 20 minutes (Figure. 2.6A), two-way ANOVA showed a potential trend for a significant interaction of injury by time ($F_{(1, 46)} = 2.93$, $p=0.09$). There were also no significant differences related to injury in time spent in the EPM open arm (Figure. 2.6B). For time spent in the centre region of the EPM (Figure. 2. 5C) there was an interaction of injury by time ($F_{(1, 46)} = 5.43$, $p<0.05$), with post-hoc comparisons revealing ACHI rats spent significantly less time in the centre of the EPM compared to sham rats at 20 minutes ($p<0.01$). Regarding distance travelled in the EPM, two-way ANOVA revealed a significant interaction of injury by time ($F_{(1, 46)} = 5.15$, $p<0.05$), with post-hoc comparisons revealing less distance travelled by ACHI rats when compared to sham rats at 20 minutes post-injury only ($p<0.05$; Figure. 2.5D).

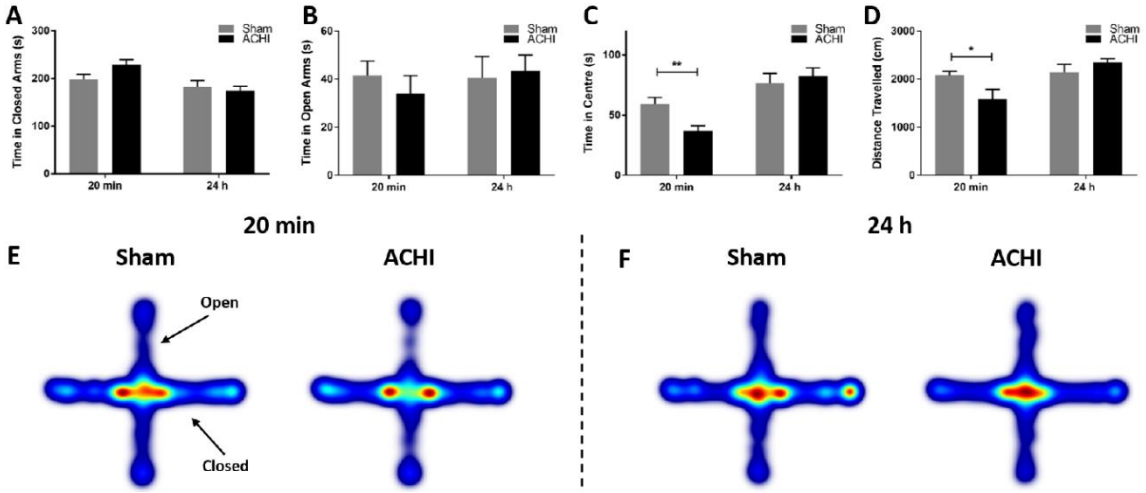

Figure. 2.6. Effect of ACHI on EPM behaviour. **(A)** There was a trend for altered duration in the closed arms between injured and sham rats (injury*time interaction, p=0.09). **(B)** No differences between sham and ACHI rats were found in time spent in open arms at both timepoints. **(C)** ACHI rats spent less time in the centre of the EPM compared to sham rats at 20 minutes (**p<0.01), with no differences apparent at 24 h. **(D)** Distance travelled within the EPM was lower in ACHI rats compared to sham rats at 20 minutes (*p=0.05), however there were no such differences at 24 h. **(E)** Grouped heatmaps of sham and ACHI rats at 20 minutes post-ACHI depicts these subtle differences in EPM behaviours. **(F)** At 24 h, grouped heatmaps between sham and ACHI rats appear similar. n=9-16 per group at each time-point (no repeated testing). Data presented as mean + SEM.

2.3.5 Lack of overt structural changes

Cresyl violet staining was used to visually examine for any overt structural changes at three discrete time-points following the administration of the ACHI. There was no evidence of significant structural damage at the injured ipsilateral side (Figure. 2.7) and no overt difference in the presence of nissl dark stained neurons (results not shown) at 1-, 3-, and 14-d following the administration of the ACHI.

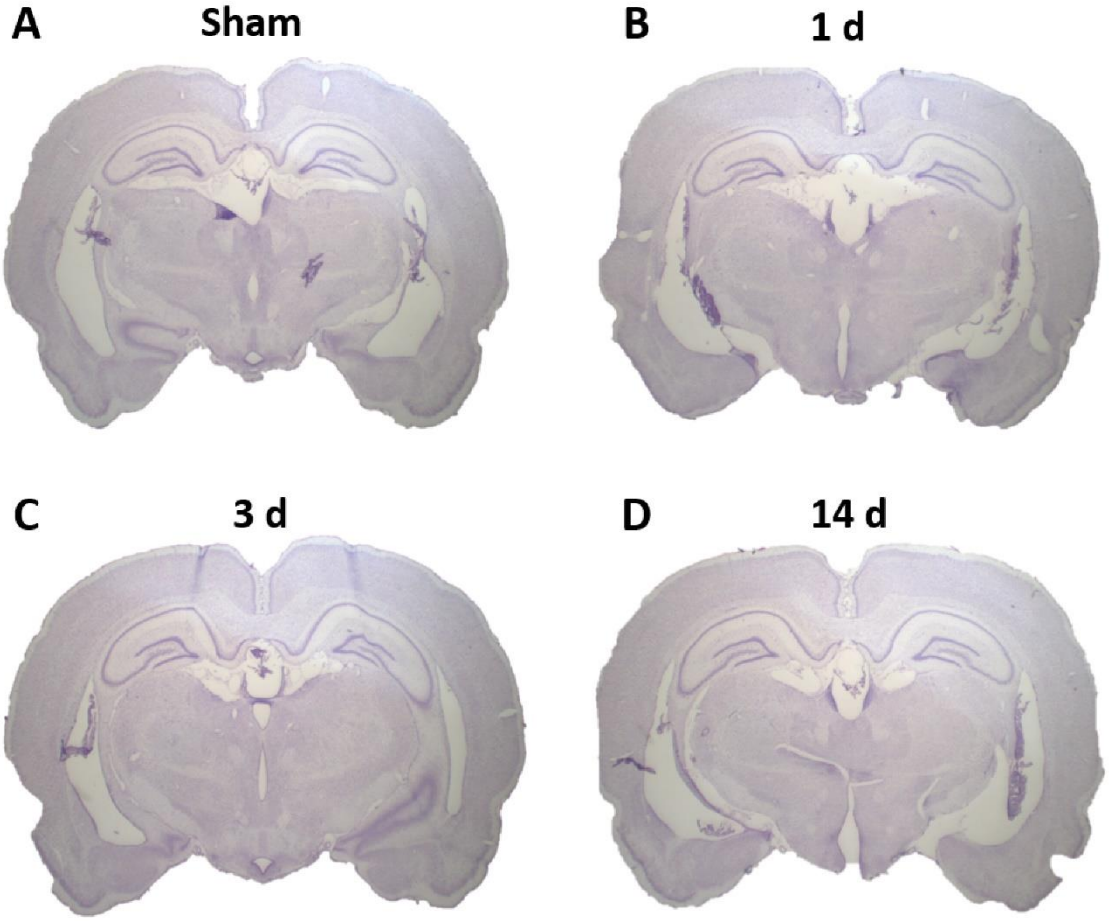

Figure. 2.7. Nissl staining of sham and ACHI brains at the site of impact. Brains were collected from sham and injured rats at 1-, 3- and 14-d post-sham/ACHI. Representative images of cresyl violet stained 30 µm coronal sections from **(A)** sham, **(B)** 1 days post-ACHI, **(C) 3** days post-ACHI, and **(D)** 14 days post-ACHI. n=3 per time-point. Low magnification images show no overt structural changes following ACHI (left side is ipsilateral injury site).

2.3.6 Microglial disturbances following ACHI

To provide insights on microglial activation, Iba-1 staining was performed on sections centred on the ipsilateral and contralateral PCX, CC and DG of sham and ACHI brains at 1-, 3- and 14-d. A main effect of injury was found for Iba-1 immunoreactivity in the ipsilateral PCX (Figure. 2.8A,G; $F_{(1,\ 29)} = 4.63$, $p<0.05$), however, there was no significant injury by time interaction nor differences detected with post-hoc comparisons.

For the ipsilateral CC there was a main effect of injury (Figure. 2.8B,H; $F_{(1, 29)} = 6.76$, $p<0.05$), with post-hoc analysis revealing increased area above threshold for Iba-1 immunostaining in the ipsilateral CC of ACHI rats when compared to sham at 3 days only (Figure. 2.8B,H, $p<0.05$), and no such differences found at both 1- and 14-d (Figure. 2.8H). There were no differences in Iba-1 labelling in the ipsilateral DG between sham and injured rats (Figure. 2.8C,I). Analysis of the contralateral PCX, CC and DG revealed no significant effects of injury on Iba-1 staining (data not shown).

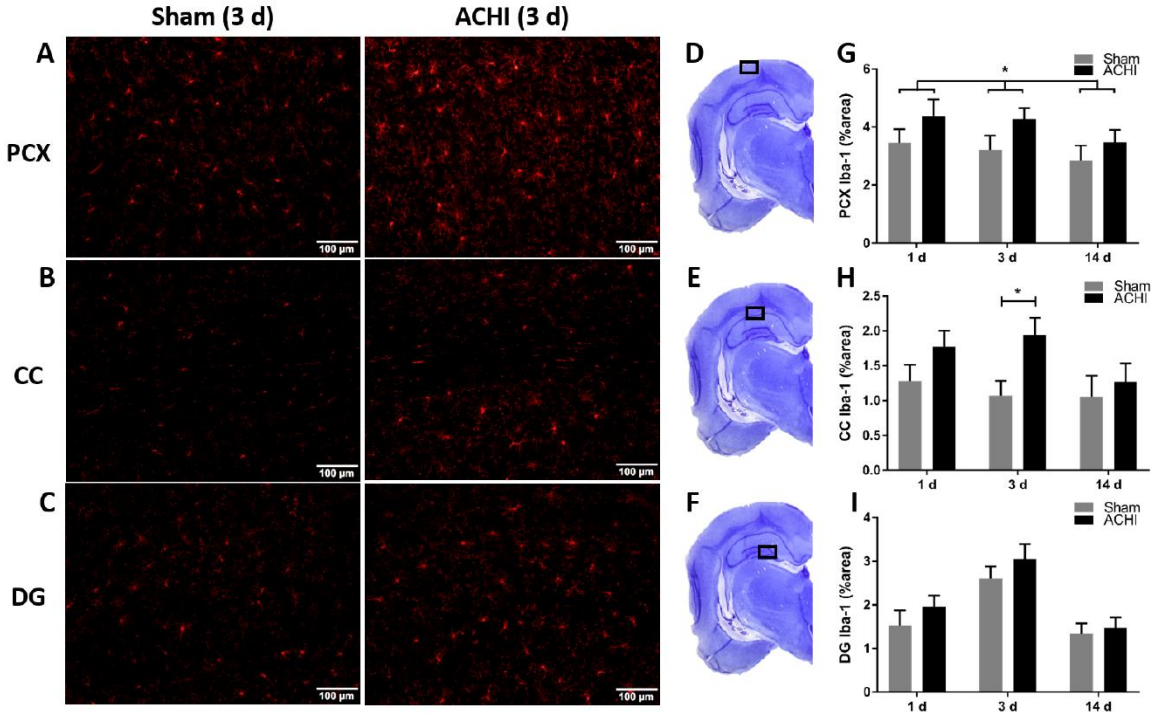

Figure. 2.8. Effect of ACHI on microglial reactivity. Brains were collected at 1-, 3- and 14-d after single sham or ACHI procedure for quantification of Iba-1 immunoreactivity. Images of three regions per sample were taken at 200x magnification. Representative images of Iba-1 immunofluorescence staining in the ipsilateral **(A)** parietal cortex (PCX)**, (B)** corpus callosum (CC), and **(C)** dentate gyrus (DG) of sham and single ACHI rats 3 days post procedure. Images were centred over **(D)** PCX **(E)** CC and **(F)** DG. **(G)** There was an overall effect of injury on Iba-1 immunoreactivity in the ipsilateral PCX (*$p<0.05$). **(H)** Percentage area of Iba-1 was also increased in the CC of ACHI rats compared to sham rats at 3 days post injury (*$p<0.05$), with no differences between

groups at 1- and 14-d. **(I)** There were no differences in Iba-1 labelling in the ipsilateral DG. n=5-7 per group at each time point. Data presented as mean + SEM.

2.3.7 Astrocytic changes following ACHI

Astrocyte activation was assessed using quantification of GFAP immunostaining. There were no observed differences in GFAP labelling in the ipsilateral PCX (Figure. 2.9A,G), with a potential trend for an injury and time interaction for GFAP staining in the ipsilateral CC (Figure. 2.9B,H; $p=0.10$). However, for the ipsilateral DG, there was a significant injury by time interaction (Figure. 2.9C,I; $F_{(2, 29)} = 3.61$, $p<0.05$), with multiple comparisons revealing a significant increase in the area above threshold for GFAP immunostaining in ACHI when compared to sham rats at 3 days post-injury ($p<0.05$), with no such differences found at 1- or 14-d (Figure. 2.8I). Analysis of the contralateral PCX, CC and DG revealed no significant effects of injury on GFAP staining (data not shown).

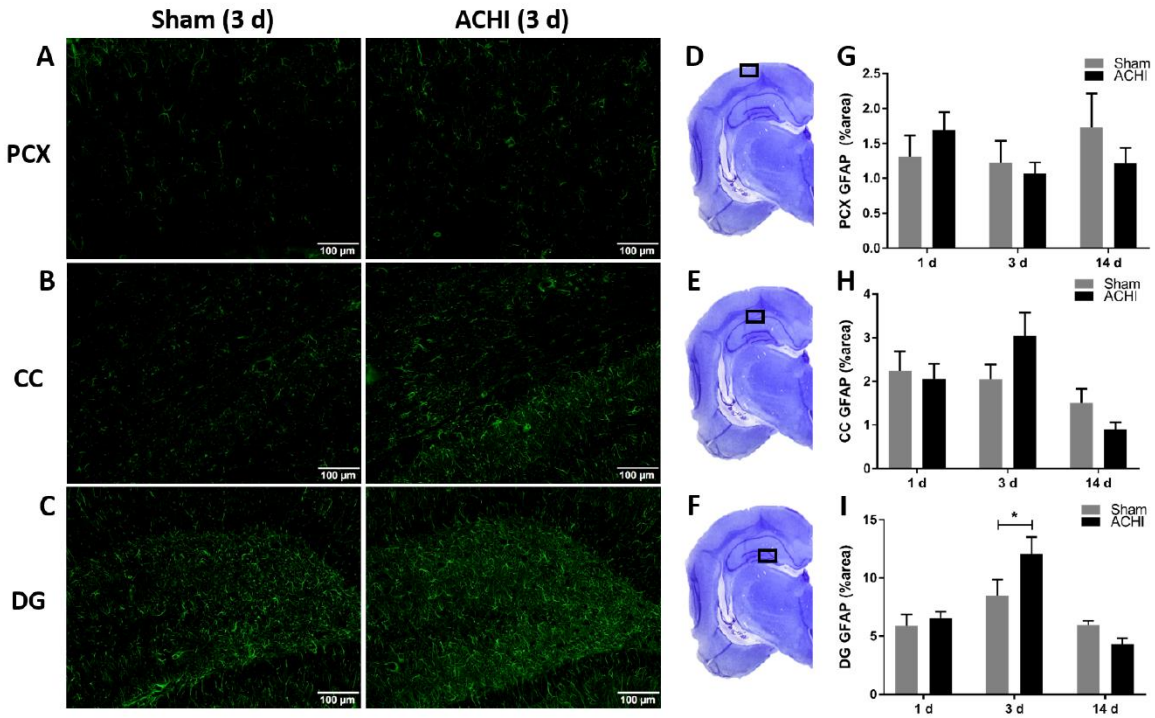

Figure. 2.9. Effect of ACHI on astrocytic reactivity. Brains were collected at 1-, 3- and 14-d after single ACHI or sham procedure for quantification of GFAP expression. Images of three regions per sample were taken at 200x magnification. Representative images of GFAP immunofluorescence staining in the ipsilateral **(A)** parietal cortex (PCX)**, (B)** corpus callosum (CC), and **(C)** dentate gyrus (DG) of sham and single ACHI rats 3 days post procedure. Images were centred over **(D)** PCX **(E)** CC and **(F)** DG. There was no significant effect of ACHI on GFAP expression in the PCX **(G)** and CC **(H)**. **(I)** GFAP labelling was increased in the DG for ACHI rats compared sham rats at 3 days (*$p<0.05$), with no differences found at 1- and 14-d. n=5-7 group at each time-point. Data presented as mean + SEM.

2.4 Discussion

Rodent models allow highly controlled, rigorous and time-efficient investigations into the effects of concussion and the underlying mechanisms. There is a growing consensus that models which have a greater degree of face validity and minimise the use of confounds may be necessary to resolve some of the most pertinent ongoing issues in this field [11, 197]. In this study, I prioritised the development of a rat model that closely replicates several features typical of clinical concussion. Our model of concussive-like injury in rats eliminates the need for anaesthesia, features a controlled blow to the un-restrained head, and most importantly, results in the rapid onset of short-lived impairments in neurological function that resolve within 24-48 h. The immunohistochemical findings indicate that a single ACHI induces no overt structural changes, but results in transient and region-specific activation of microglia and astrocytes. This glial activation was found to peak at 3 days post-impact, indicating that other neurobiological mechanisms likely underlie the acute behavioural impairments.

2.4.1 Nature of neurobehavioural findings

Beam testing revealed that when compared to sham rats, those given a single ACHI had significant sensorimotor impairment at 1-2 minutes post-impact. Notably, all 15 ACHI rats had an increased number of hind-limb slips immediately post-impact when compared to their baseline performance, indicating a reliable effect of the impact on this task. Importantly, beam deficits had resolved by 24 hours post-impact, suggesting that, as often seen in clinical concussion [6, 221], sensorimotor deficits were relatively short-lived. Additional testing is required to understand the mechanisms underlying these changes, though they likely reflect temporary disturbances to one or more sensorimotor pathways, such as those involved in balance, motor control or vision. Furthermore,

although our findings suggest acute and transient changes, it is possible that more subtle sensorimotor deficits (e.g. gait alterations) may linger beyond 24 hours post-impact.

Our Y-maze findings indicated that cognitive deficits were also present in the acute stages post-ACHI; however, these impairments persisted beyond the other detected behavioural changes, with differences in novel arm preference also found between sham and ACHI rats tested at 24 hours post-injury. In rats tested at 48 hours post-impact, a novel arm preference was found in both sham and ACHI rats, indicating no differences in spatial memory at this time-point. Taken together, these findings indicate that ACHI induced transient deficits in short-term spatial memory, with the duration of these impairments lasting beyond the other detected behavioural changes. Though this result may represent potential differences in behavioural task sensitivity, it does align with a number of clinical studies that have reported a relative lag in cognitive recovery compared to other clinical symptoms of concussion [6, 222, 223]. Although the Y-maze protocol was consistent between time-points, it is important to recognise that rats in the acute testing group were exposed to the two arms of the apparatus at 1 hour prior to sham/ACHI, whereas rats in the other recovery groups were firstly exposed after sham/ACHI, i.e. 1 hour prior to testing at either 24- and 48-h. As such, our findings likely reflect a degree of retrograde amnesia at 5 minutes post-impact, and anterograde amnesia at 24 hours post-impact. Further studies are required to determine the exact nature and mechanisms of these memory deficits; however, given the location of the impact site and our immunohistochemical findings, it is likely that hippocampal disturbances are involved.

We hypothesised that ACHI would result in altered anxiety-like behaviour, as reported in other models of mTBI [224-226] and clinical concussion [227, 228]. Although there were no significant differences between sham and ACHI rats in EPM arm duration

at any time-point in this study, there was a potential trend (injury*time, p=0.09) for injured rats to spend more time in the closed arm at 20 minutes post-injury, as well as a significant reduction in time spent in the centre zone of the EPM and total distance travelled by ACHI rats at this time-point. Although these findings indicate that ACHI significantly altered behaviour in the EPM, it is not possible to conclude that these changes are anxiety-related. It is possible that anxiety-related changes may occur at other time-points post-ACHI. Further, it is also possible that beam and OF testing prior to EPM confounded these results; however, some laboratories have reported minimal effects of behavioural test batteries on EPM performance [229], and in fact, there is some evidence that pre-exposure to a novel environment (e.g. OF) shortly before EPM testing increases motor activity and the likelihood of entering the EPM open arms [229, 230], potentially increasing the likelihood of detecting anxiolytic behaviour. Another factor that may limit interpretation of the anxiety-related behaviour in the EPM is our finding of reduced distance travelled in the EPM by ACHI rats at 20 minutes post injury.

Although there were no differences in distance travelled in the Y-maze or OF at 5- and 10-minutes respectively, the significant reduction in distance travelled by ACHI when compared to sham rats in the EPM at 20 minutes post-impact is a point of interest. The contrasting findings in distance travelled between these tasks likely reflects either a time- or task-dependent effect of injury on locomotor activity. It is possible that several symptoms induced by ACHI contributed to the reduced exploration of the EPM, including motor impairments, fatigue, drowsiness or nausea. Regardless of the underlying mechanisms, this finding further demonstrates that a single ACHI induced transient neurobehavioural disturbances.

2.4.2 Transient, region-specific glial reactivity following ACHI

Immunofluorescence staining for Iba-1 and GFAP revealed region-specific increases in glial reactivity ipsilateral to the impact site, peaking at 3 d. Increases in labelling for microglial/macrophage marker Iba-1 were most pronounced around the CC, whereas astrocytic marker GFAP was found to increase in the DG. These findings likely reflect an increase in reactive microglia and astrocytes [231-234]. Interestingly, I observed no differences in expression of these glial reactivity markers at 1 days post-ACHI, indicating that the persistent Y-maze deficits I observed at this time-point were likely due to other neurobiological changes induced by impact. In addition, our findings of neurobehavioural resolution prior the detected peak in glial reactivity adds to the growing evidence that symptom resolution may precede neurobiological recovery from concussive injury [188, 235]. Future studies will investigate how this seemingly delayed-onset but transient increase in glial reactivity may contribute to the potential temporal window of ICV to exacerbated or persistent effects of repeated concussions.

2.5 Conclusions

This study investigated the temporal changes in neurobehaviour, and glial reactivity induced by a novel model of concussive-like injury in conscious rats. The elimination of anaesthetic and surgical procedures in our high-throughput ACHI model not only removes the potential confounds these factors may have on pathophysiology, but significantly, allowed for detailed neurobehavioural assessments to be made within 20 minutes of the impact. Importantly, the findings from this study demonstrated that a single ACHI induced the rapid onset of neurobehavioural impairments, all of which had resolved within 24-48 h. Our immunohistochemical findings indicate that glial reactivity peaked at 3 days post-impact, notably after the time-point of neurobehavioural resolution.

Such temporal characterisation of the effects of a single mTBI is often overlooked, but is of particular importance when attempting to mimic concussion in humans. In particular, although a number of recent rodent-based studies have demonstrated cumulative and potentially chronic effects of repeated concussive-like injuries, the clinical relevance of repeated impacts delivered prior to a defined time-point of symptom resolution is questionable, particularly given the recent improvements in clinical concussion management (e.g. return to play guidelines). I postulate that prioritisation of the clinical relevance of both the animal model and study design is necessary to increase our understanding of the neurobiological basis and effects of concussions, and as such, to provide new insights that may lead to improved clinical management.

Chapter 3: Behavioural, axonal, and proteomic alterations following repeated mTBI: Novel insights using a clinically relevant rat model

The information presented in this chapter has been published prior to the completion of this thesis. The full PDF copy of this manuscript is attached in **Appendix E**. Author contribution state can be found in **Appendix B**. Copyright permissions can be found in **Appendix C**.

Journal article: **Pham, L.,** Wright, D. K., O'Brien, W. T., Bain, J., Huang, C., Sun, M., Casillas-Espinosa, P. M., Shah, A. D., Schittenhelm, R. B., Sobey, C. G., Brady, R. D., O'Brien, T. J., Mychasiuk, R., Shultz, S. R., & McDonald, S. J. (2020) Behavioral, axonal, and proteomic alterations following repeated mild traumatic brain injury: Novel insights using a clinically relevant rat model. *Neurobiology of Disease*, Epub ahead of print.

3.1 Introduction

Mild traumatic brain injuries (mTBIs), such as concussions, account for >80% of TBI cases [3]. mTBI is particularly common in sports with the highest participation rates for adolescents and young adults [4, 192]. mTBI is also increasingly prevalent in military personnel (e.g. warzone blast exposure) [236, 237], and is a leading cause of emergency department visits [238, 239]. Although the neurobehavioural (e.g. cognitive and emotional impairments) and physical (e.g., headache, motor deficits) effects of mTBI most often resolve within 7-10 days, approximately 20% of individuals are plagued by symptoms that persist for several weeks, months or longer (i.e. PPCS) [240]. There is mounting evidence that a history of mTBI may be the strongest risk factor for sustaining a future mTBI, and also for experiencing more symptoms for a longer duration should another mTBI occur [7, 148, 241]. Risk of exacerbated symptoms is thought to be particularly high if mTBIs are experienced in short succession [150, 242]. Moreover, several recent retrospective studies on individuals with a history of mTBI or repetitive head trauma have provided evidence of an increased risk for long-term neurological conditions, such as depression and cognitive impairment [194], or progressive neurodegenerative diseases such as Alzheimer's and CTE [161, 243]. However, there is still a great deal of controversy and debate surrounding the associations between repeated

mTBI and persistent or progressive neurological conditions [163, 244], and this topic requires further investigation.

The last two decades have seen a significant increase in understanding of the neuropathology that may underlie the effects of repeated mTBIs. Post-mortem analyses of individuals with an extensive history of mTBI have found evidence of microstructural pathology, including widespread axonal disruption and proteopathies, and in some cases, significant atrophy of certain brain regions [245, 246]. Clinical *in vivo* neuroimaging studies have also provided evidence of reduced white matter integrity [247, 248], tau pathology [177, 249] and microglial activation in people with a history of repeated mTBI [177, 178]. Nonetheless, the retrospective nature and presence of confounding variables in many of these studies make it difficult to decipher the true prevalence and magnitude of these neuropathological changes. Moreover, with most studies conducted at single time-points several years or decades following the final injury, the temporal progression of neuropathology is poorly understood.

Animal models of repeated mTBI have allowed for important neuropathological insights not possible in clinical studies. Multiple rodent studies have found that repeated mTBIs can trigger significant and potentially lasting changes in energy metabolism, blood brain barrier damage, oxidative stress, neuroinflammation, axonal damage, tau pathology, and neurodegeneration [146, 172, 181, 250-254]. Nevertheless, the clinical relevance of some animal models used to create this understanding has been questioned, with anaesthetics or craniotomy both shown to alter TBI pathophysiology and outcomes, and some models producing structural changes (e.g. cavitation) not typically seen in clinical mTBI [129]. Moreover, although clinical and pre-clinical studies have increased understanding of the potential cellular and molecular consequences of repeated mTBI, a

number of important questions remain, particularly regarding the progression of these changes through the acute and chronic stages of injury.

To provide further insights into the pathophysiological and neurobehavioural effects of mTBIs, I have recently co-developed and characterised an awake, closed-head injury (ACHI) model in rats. **Chapter 2** found that a single injury with this anaesthetic- and surgery-free model results in transient neurobehavioural symptoms that typically resolve within 24-48 hours post-injury, and transient increases in glial reactivity that appear to resolve within two weeks. Repeated ACHIs have also recently been shown to induce acute changes in white matter that resemble those seen in clinical mTBI [255]. In the current study, I aimed to determine the acute and chronic effects of repeated ACHIs separated by 48 h. To do so, I implemented a detailed neurobehavioural battery to characterise the extent and evolution of neurobehavioural changes, blood (NfL) and neuroimaging (DTI) biomarkers to assess axonal injury, and high-resolution mass spectrometry to investigate the hippocampal proteome at both acute and chronic stages after repeated mTBI.

3.2 Materials and Methods

3.2.1 Animals

Sixty-four male Long-Evans rats were obtained from Animal Resource Centre (WA, Australia) at PND 28. Rats were housed in pairs at the La Trobe University Central Animal House, with a 12h:12h light/dark cycle and food and water available *ad libitum*. All procedures were performed in accordance with the Animal Ethics Committee at La Trobe University (AEC 17-06) and were within the guidelines of the Australia code of practice for the use of animals for scientific purposes by the Australian National Health

and Medical Research Council. All animals were allowed 48h to acclimatise to their enclosures upon arrival before experimenter contact. After this period animals were handled 4 days per week until the first sham or injury procedure on PND 40-42.

3.2.2 Mild TBI

Rats were randomly allocated into groups that received four sham injuries or four ACHI procedures, with each procedure separated by 48 h. This injury interval was chosen as we previously found behavioural deficits at 48 hours post-ACHI in **Chapter 2**. Sham and ACHI procedures were completed similar to those described in **Chapter 2**. Briefly, a steel helmet was placed over the Decapicone™-restrained rat (Braintree Scientific, MA, United States) and centered over the left parietal bone, with the rat placed on a foam bed and lightly held in position by the lower body. Once positioned, a 5 mm tip attached to a controlled cortical impactor (Leica Biosystems, IL, United States) was triggered and impacted the helmet (velocity: 6.5 m/s, depth: 8 mm, dwell time: 100 ms). The total duration of the procedure was less than 1 min. Sham procedures were performed identically to ACHIs, with the impact instead triggered adjacent to the animal's head. No skull fractures were observed during post-mortem analysis. The lack of anaesthesia allowed for inspection of visual signs that may be associated with LOC (e.g. apnea, absence of hindlimb withdrawal reflex, lying motionless). Incidence of apparent transient LOC increased after each ACHI (1st ACHI, 25.8%; 2nd ACHI, 35.5%; 3rd ACHI, 45.2%) before slightly decreasing at the 4th ACHI (29.0%). Although these measures were conducted by an experimenter blinded to the experimental condition, it should be acknowledged that assessment of LOC is inevitably a subjective measure in this context. Once rats had self-righted and were mobile (typically within 1-minute post-injury), the beam task was used to assess sensorimotor coordination for all rats for each procedure (as described below). One rat was excluded prior to injury due to a skin wound sustained in

its home cage. Rats were assigned into either acute 7-day or chronic 3.5-month recovery groups.

3.2.3 Neurobehavioural testing

All rats performed beam task immediately post-each injury. Rats assigned to seven-day recovery (i.e. acute) performed open field (24 hours following final sham/ACHI), Y-maze (two days), EPM (three days) and beam task (three days). To avoid repeated testing, rats assigned to chronic recovery were exposed to the same tasks beginning at three months, with the addition of water maze. All behavioural assessments, excluding beam task (recorded with a stationary camera), were recorded by an overhead camera and analysed using tracking software Ethovision (Ethovision XT 10; Noldus, the Netherlands). With the exception of the beam task conducted immediately post-injury, experimenters were blinded to the rat group at all stages during testing. All video/data analysis was conducted by a researcher blinded to the experimental conditions.

Sensorimotor function was assessed via the beam task at baseline one hour prior to the first injury, and one minute post each procedure for all recovery groups similar to that described previously [212]. The acute recovery rats repeated the task at three days following the final sham/ACHI, and chronic rats 3.5 months. The task consisted of a 2 cm wide, 1.5 m long wooden beam, elevated 75 cm above the ground, with a protective mattress placed below to soften any falls. At the start-end of the beam there was a bright light (i.e. an aversive stimulus), as well as a camera to record the task. At the finish-end, a dark "home box" was placed with home cage bedding to encourage completion of the task. Prior to testing, beam training took place as previously described in **Chapter 2**. Rats were required to traverse 1.0 m on the beam to the home box to complete a trial, with five trials used for each session. In a small number of cases, trials were re-performed if rats were immobile for longer than five sec. Average latency to cross the beam and hindlimb

slips were later determined through captured video analysis by a researcher blinded to the experimental conditions. Rats that failed to complete a trial within 20 seconds were assigned this value. Rats that fell were automatically given this maximum time as well as 2 hindlimb slips.

Locomotion/activity was assessed in an OF similar to that described previously [215]. This task consisted of a 100 x 100 cm sawdust-covered arena, with 40 cm high walls to prevent escape. Rats began in the center of the field and were allowed to explore for five minutes before they were returned to their home cage. Locomotion was assessed by total distance travelled. To measure anxiety, the arena was divided into an inner zone (66 x 66 cm^2) and outer zone and the time spent in the inner zone was determined.

The previous chapter found that a single ACHI induced spatial memory deficits in the Y-maze at 24 h, with resolution apparent at 48 h. Therefore, the current study used Y-maze to assess spatial memory, similar to that described previously [220], 48 hours after the final injury in the acute recovery groups, as well as at 3.5 months in the chronic recovery groups. Rats were introduced into a Y-maze for a 10-minutes exploration phase, with the novel arm closed, then returned to their home cage. One hour later, rats were reintroduced to the Y-maze for a 5 minutes testing phase with all arms open. Entries and time spent in the novel arm and familiar arm were recorded and used to generate a discrimination preference index for each measure. The discrimination index was calculated as follows: (novel arm - familiar arm) / (novel arm + familiar arm) [256].

Anxiety-like behaviour was measured using an EPM similar to that described previously [257]. The plus-shaped maze platform was elevated 50 cm above ground and consisted of two open and two closed arms (50 x 10 cm/arm) and a square (10 x 10 cm) central platform. Rats were placed into the center of the EPM and allowed to explore the arms of

the maze for 5 minutes before returning to their home cage. Duration spent in the open arms was quantified.

After the completion of other behavioural tasks, spatial cognition of chronic recovery rats was assessed using water maze similar to as previously described [181]. A 163 cm diameter circular pool was filled with tap water (28 ± 1°C). Non-toxic white paint (150 ml) was used to opacify the water and create contrast for rat tracking. The arena was divided into four quadrants, north-east (NE), north-west (NW), south-east (SE) and south-west (SW). A circular 10 cm diameter acrylic escape platform was submerged 2 cm below the water surface in the center of a randomised quadrant. Testing consisted of two days; acquisition (day 1) and reversal (day 2) consisting of 10 trials per day with a maximum time of 60 seconds per trial. Trials began when rats were placed into the pool facing the wall at one of eight pseudorandomised locations (N, S, E, W, NE, NW, SE, SW) and required to swim to locate and stand on the hidden platform to be 'rescued' by the experimenter. If the maximum time elapsed, rats were led to the hidden platform by the experimenter and left for 15 seconds before being 'rescued'. Settings for reversal were the same as for acquisition, with the exception of the hidden platform, which was placed in the opposite quadrant before trials commenced. Average latency to platform, percentage of time in target quadrants and percentage of direct and circle swims were calculated.

3.2.3 Tissue and Blood collection

Rats were euthanised for collection of fresh or fixed tissue at either seven days post- or 3.5 months post-last injury. To obtain fresh tissue, rats were anaesthetised with Lethobarb and culled for collection. The ipsilateral cortex and hippocampus were flash frozen in liquid nitrogen and stored at -80°C before analysis. Blood was collected by cardiac puncture and transferred to 3.5 ml Becton Dickinson Vacutainer® SST™ II Advance blood

collection tubes (Franklin Lakes, NJ, United States), and allowed to clot at room temperature for 30 min. Following centrifugation at 1500 g for 10 min, serum was snap frozen in liquid nitrogen and stored at -80°C until analysis. Chronic recovery rats were anaesthetised with Lethobarb, and following cardiac blood collection, were transcardially perfused with PBS followed by 4% PFA in PBS before brains were removed and post fixed with PFA at 4°C overnight. Samples were then transferred to 1x PBS and washed twice daily for 3 days on a rocker at 4°C and stored in 1x PBS at 4°C before they were prepared for magnetic resonance imaging (MRI).

3.2.4 Serum NfL quantification

Quantification of serum NfL was performed using a 'Simoa® NF-light Advantage Kit' run on the Simoa HD-X Analyser (Quanterix, Billerica, MA, USA). A single assay was performed on eight randomly selected samples per group, and was run in a temperature-controlled laboratory by an experimenter blinded to the experimental conditions. Samples were tested in duplicate, with a total serum volume for each sample of 106 µl. All samples measured above the lower limit of quantification for NfL (0.174 pg/ml).

3.2.5 High-resolution, data-independent acquisition mass spectroscopy

The ipsilateral hippocampus was chosen for proteomic analysis, my previous chapter found evidence of hippocampal gliosis using this ACHI model. Flash frozen ipsilateral hippocampus samples were homogenised in liquid nitrogen and lysed in 4% SDS, 100 mM HEPES, pH 8.5, which was then heated at 95°C for 5 minutes and sonicated three times for 10 seconds each at an amplitude of 10 µm. The lysates were clarified by centrifugation at 16,000 g for 10 minutes and the protein concentration was determined using the Pierce™ bicinchoninic acid Protein Assay Kit (Thermo). Equal protein amounts were denatured and alkylated using Tris(2-carboxyethyl)-phosphine-

hydrochloride (TCEP) and 2-Chloroacetamide at a final concentration of 10 mM and 40 mM, respectively, and incubated at 95°C for 5 min. SDS was subsequently removed by chloroform/methanol precipitation. Sequencing grade trypsin was added at an enzyme to protein ratio of 1:100 and the reaction was incubated overnight at 37°C. The digestion reaction was stopped by adding formic acid to a concentration of 1%. The samples were cleaned up with BondElut Omix Tips (Agilent) and concentrated in a vacuum concentrator prior to analysis by mass spectrometry.

Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap column (100 µm x 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 µm x 50 cm, nanoViper, C18, 2 µm, 100Å; Thermo Scientific). The peptides were separated by increasing concentrations of 80% ACN / 0.1% FA at a flow of 250 nl/minute for 158 minutes and analysed with a QExactive HF mass spectrometer (Thermo Scientific) operated in data-independent acquisition (DIA) mode. Sixty sequential DIA windows with an isolation width of 10 m/z have been acquired between 375 - 975 m/z (resolution: 15.000; AGC target: 2e5; maximum IT: 9 ms; HCD Collision energy: 27%) following a full ms1 scan (resolution: 60.000; AGC target: 3e6; maximum IT: 54 ms; scan range: 375-1575 m/z).

3.2.6 Mass spectrometric data analysis

The acquired DIA data have been evaluated in Spectronaut 13 Laika (Biognosys) using an in-house generated spectral library derived from rat brain samples. Of note, potential plasma protein contaminants have been removed from this library using an in-house generated spectral library derived from rat plasma samples in combination with homologs identified in the Human Blood Atlas [258]. Missing values have been imputed using background signals, protein intensities were $\log_2$ transformed and median

normalised, and two pairwise comparisons (7-day ACHI vs. 7-day sham and 3.5 months ACHI vs. 3.5 months sham) were carried out. Protein fold changes and FDR adjusted p-values (q-values) were calculated. GO terms associated with differentially regulated proteins (i.e. those with q-value <0.05 were extracted from String database [259].

3.2.7 DTI

3.2.7.1 Ex Vivo Magnetic Resonance Imaging Acquisition

Ex vivo Magnetic Resonance Imaging (MRI) was performed using a 9.4 T Bruker (Bruker™ BioSpin®, USA) and actively decoupled volume transmit and four-channel cryogenically-cooled surface receive coils. Whole fixated brains were embedded with 3% agar in 50 ml Falcon tubes for scanning. DTI was performed in 81 directions with diffusion duration (δ) = 4.5 ms, diffusion separation (Δ) = 13.5 ms and b-value = 4000 s/mm^2. We also obtained one non-diffusion (b_0) volume. Image parameters also included repetition time (4,500 ms), echo time (34 ms), field of view (32 x 24 mm^2), 48 slices of 250 µm thickness and matrix size (128 x 96) giving an isotropic spatial resolution of 250 x 250 x 250 µm^3.

3.2.7.2 MRI processing and analysis

MRtrix3 (www.mrtrix.org) was used to process DTI [260]. DTI metrics including FA, axial diffusivity, RD and the apparent diffusion coefficient were calculated and registered to a study-specific template as described previously [261]. Whole-brain voxel-wise analysis was performed using tract-based spatial statistics (TBSS) with threshold free cluster enhancement (TFCE).

ITK-SNAP [262] was used to manually trace regions of interest (ROI) on seven consecutive coronal MRI T_2-weighted structural images. ROIs included the cortex, CC and hippocampus and the total volume of each ROI was calculated using ITK-SNAP.

3.2.8 Statistical analysis

All outcomes were analysed using GraphPad Prism version 8.3.1 for Windows (GraphPad Software, CA). Water maze percentage time in target quadrant, direct and circle swims were analysed using unpaired t-test and presented as mean + standard error of the mean. For correlation analysis of serum NfL levels and beam performance, Pearson r was used to assess normally distributed data, while Spearman r was used to assess data not normally distributed. Permutation testing of DTI metrics was performed using FSL's *randomise* with 5,000 permutations and fully corrected for multiple comparisons with TFCE. Two-way repeated measures ANOVA were used for beam task analysis immediately after injury. All other data was analysed with two-way ANOVA, with variables of injury and time. Sidak's multiple comparisons performed where appropriate. Statistical significance was accepted if $p \leq 0.05$. Due to potential violations of assumptions of normality for beam data, additional analysis was performed for beam hindlimb slips with Mann Whitney tests (corrected for multiple comparisons, alpha 0.025) and beam traverse time data was transformed for 3 d and 3.5 mo before two-way ANOVA analysis.

3.3 Results

3.3.1 ACHI resulted in sensorimotor deficits immediately post-injury

Beam task was used to assess sensorimotor function at baseline and one minute after each sham/injury procedure (n=31/group; Figure. 3.1). The average time taken to traverse the beam one minute after each ACHI (Figure. 3.1A) was analysed using repeated measures two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)}=60.27$; $p<0.0001$) and time ($F_{(4, 240)}=8.543$; $p<0.0001$), and a significant interaction of injury by time ($F_{(4, 240)}=13.88$; $p<0.0001$). *Post hoc* analysis found repeated ACHI rats took

significantly longer to traverse the beam compared to sham rats after each ACHI procedure (Figure. 3.1A; p<0.0001 for all time-points). Total number of hindlimb slips 1 minutes after each ACHI (Figure. 3.1B) was analysed using two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)}$=114.0; p<0.0001) and time ($F_{(4, 240)}$=18.74; p<0.0001), and a significant interaction of injury by time ($F_{(4, 240)}$=18.51; p<0.0001). *Post hoc* comparisons found that injured rats displayed significantly more hindlimb slips at 1 minutes after each ACHI compared to sham rats (Figure. 3.1B; p<0.0001 for all time-points).

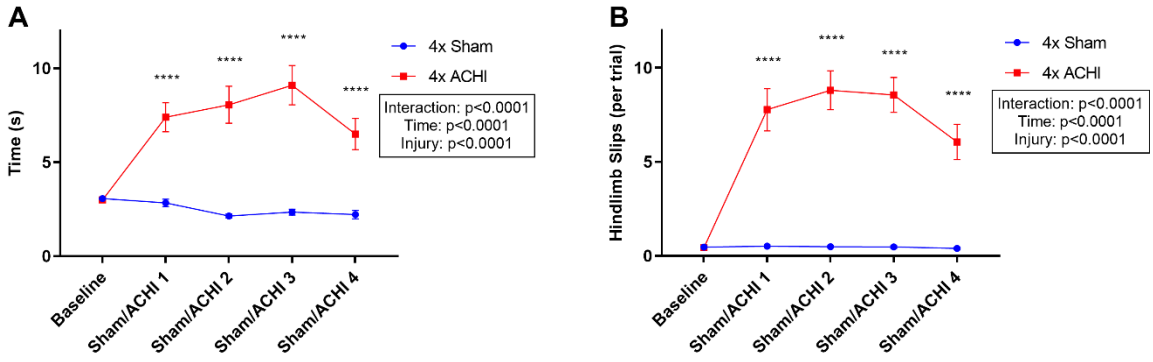

Figure. 3.1. Effect of repeated ACHI on sensorimotor function immediately after each injury. **A)** At 1 minute after each ACHI, rats took significantly longer to traverse the beam compared to sham rats (ACHI 1-4, each ****p<0.0001). **B)** Repeated ACHI rats also displayed more hind-limb slips compared to repeated sham rats (ACHI 1-4, each ****p<0.0001). n=31/group at each time-point. Data presented as mean ± SEM

3.3.2 Repeated ACHI resulted in persistent sensorimotor deficits

Beam task was also used to assess sensorimotor deficits at three days (n=14-15/group) and 3.5 months (n=17/group) after the final injury (Figure. 3.2). One rat, allocated to the long-term recovery group, completed beam task at 3 days and therefore was included for this analysis only. For time taken to traverse the beam three days and 3.5 months after final-sham/ACHI (Figure. 3.2A), a two-way ANOVA was performed after

excluding one rat with incomplete data (video recording error), and revealed a main effect of injury ($F_{(1, 59)}$=6.44; p=0.014), and time ($F_{(1, 59)}$=12.50; p<0.001), with no interaction of injury and time ($F_{(1, 59)}$=0.86; p=0.356). For hindlimb slips (Figure. 3.2B) there was a main effect of injury ($F_{(1, 59)}$=4.04; p=0.049), but not for time ($F_{(1, 59)}$=0.14; p=0.709), or an injury by time interaction ($F_{(1, 59)}$=1.82; p=0.182).

Additional analyses was performed due to potential violations of assumptions of normality for beam data analysed with linear models. Additional analysis of beam slips was performed using Mann Whitney tests (corrected for multiple comparisons, alpha=0.025), with analysis of 3 d data revealing a significant difference between sham and repeat ACHI rats (p=0.015), and no differences at 3.5 mo (p=0.625). Two-way ANOVA analysis of natural log (Ln) transformed beam traverse data revealed a main effect of injury ($F_{(1, 59)}$=6.440, p=0.0138), along with a main effect of time ($F_{(1, 59)}$=12.50, p=0.0008), and no injury x time interaction ($F_{(1, 59)}$=0.8641 p=0.356).

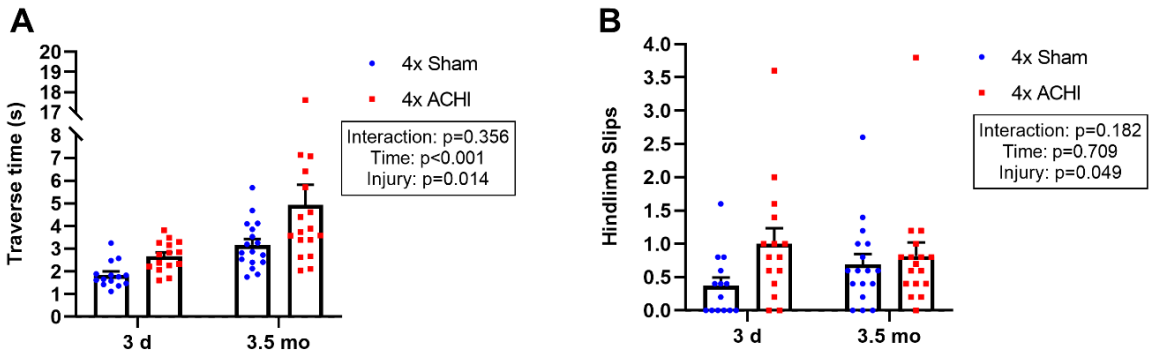

Figure. 3.2. Effect of repeated ACHI on sensorimotor function at 3 days and 3.5 months after final ACHI. **A)** A main effect was found in injury (p=0.018) for beam traverse time at 3 days and 3.5 months after final-sham/ACHI **B)** For hindlimb slips per trial at 3 days and 3.5 months post-final ACHI, there was a main effect of injury (p=0.048). n=30-31/group at baseline, n=14-15/group at 3 days, n=16-17/group at 3.5 months. Data presented as mean + SEM

3.3.3 No differences in locomotion and anxiety-related behavioural

Locomotor activity and anxiety-like behaviour were assessed using open field at one day (n=14/group) and 3.5 months (n=17/group) following repeated sham/injury, with no differences found in total distance travelled (Figure. 3.3A) and time spent in center zone (Figure. 3.3B).

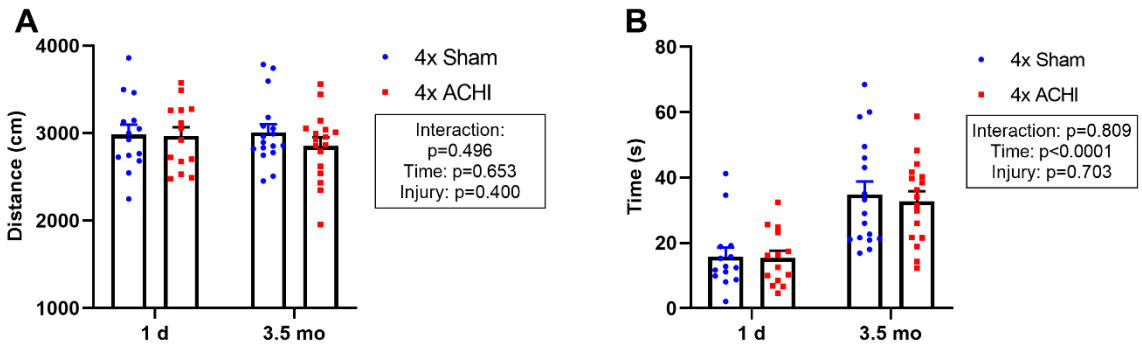

Figure. 3.3. Locomotor and anxiety-like behaviour after repeated ACHI. Open field revealed no differences between repeated sham ACHI rats in **A)** distance travelled and **B)** time spent in centre at both 1 day and 3.5 months after final repeated sham/ACHI. n=14-17/group at each time-point. Data presented as mean + SEM.

EPM was used to assess anxiety-like behaviour at three days (n=14/group) and 3.5 months (n=17/group) after repeated sham/ACHI (Figure. 3.4). For the duration of time spent in the open arms there was a main effect of time ($F_{(1, 58)}$=30.57; p<0.0001), however there was no main effect of injury ($F_{(1, 58)}$=0.00; p=0.980) and no injury by time interaction ($F_{(1, 58)}$=0.03; p=0.853).

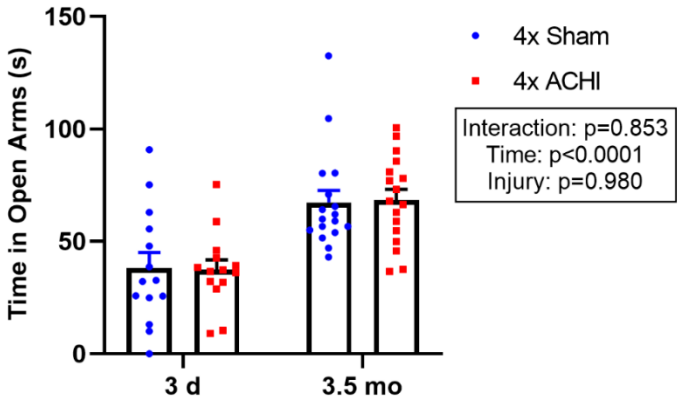

Figure 3.4. Effect of repeated ACHI on anxiety-like behaviour. No differences between repeated sham ACHI rats were found in time spent in open arms at both 3 days and 3.5 months after final repeated sham/ACHI in the elevated plus maze. n=14-17/group at each time-point. Data presented as mean + SEM.

3.3.4 Impaired spatial memory at acute, but not chronic, stages post-ACHI

The effect of repeated ACHI on spatial memory of the Y-maze was assessed at two days (n=13/group) and 3.5 months (n=16-17/group) post-final sham/ACHI (Figure. 3.5). Rats were excluded if they spent less than 10 seconds in each arm (n=2) or escaped the maze (n=1). For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury ($F_{(1, 55)}$=5.04; p=0.029), however, no main effect of injury ($F_{(1, 55)}$=1.26; p=0.267) or time ($F_{(1, 55)}$=0.72; p=0.399). *Post hoc* analysis found a trend for repeatedly injured rats to spend less time in the novel when compared to the familiar arm at 2 days (p=0.056) but not at 3.5 months post-final injury compared to sham rats. Entries into the novel/familiar arms between sham and repeated ACHI rats was also assessed, revealing no main effect of injury ($F_{(1, 55)}$=0.60; p=0.442), time ($F_{(1, 55)}$=3.02; p=0.088), or injury by time ($F_{(1, 55)}$=2.34; p=0.132).

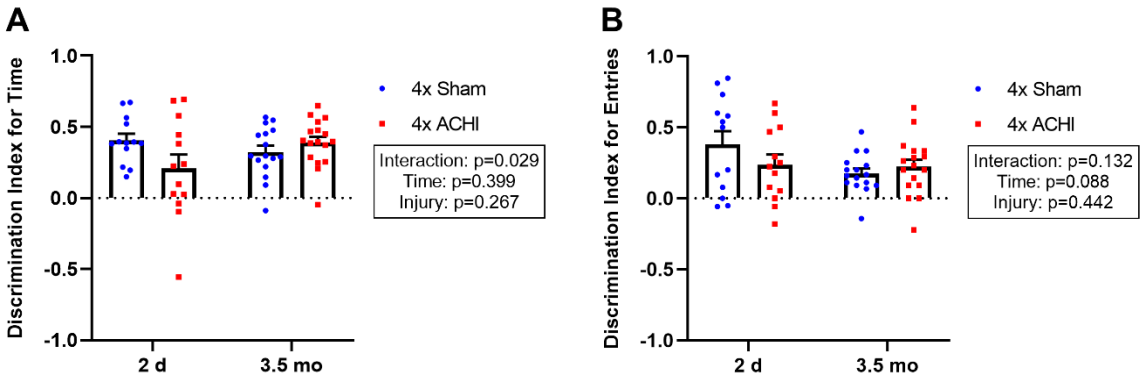

Figure. 3.5. Effect of repeated ACHI on spatial memory. **A)** For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury **B)** No significant differences were observed in novel arm entries between repeated ACHI and sham rats. n=13-17/group. Data presented as mean + SEM.

Spatial memory at the chronic timepoint was also assessed using water maze (n=17/group). In both acquisition and reversal testing, no differences were seen for latency to reach the platform, time in target quadrant, time in previous target quadrant (reversal), and direct and circle swims (Figure. 3.6).

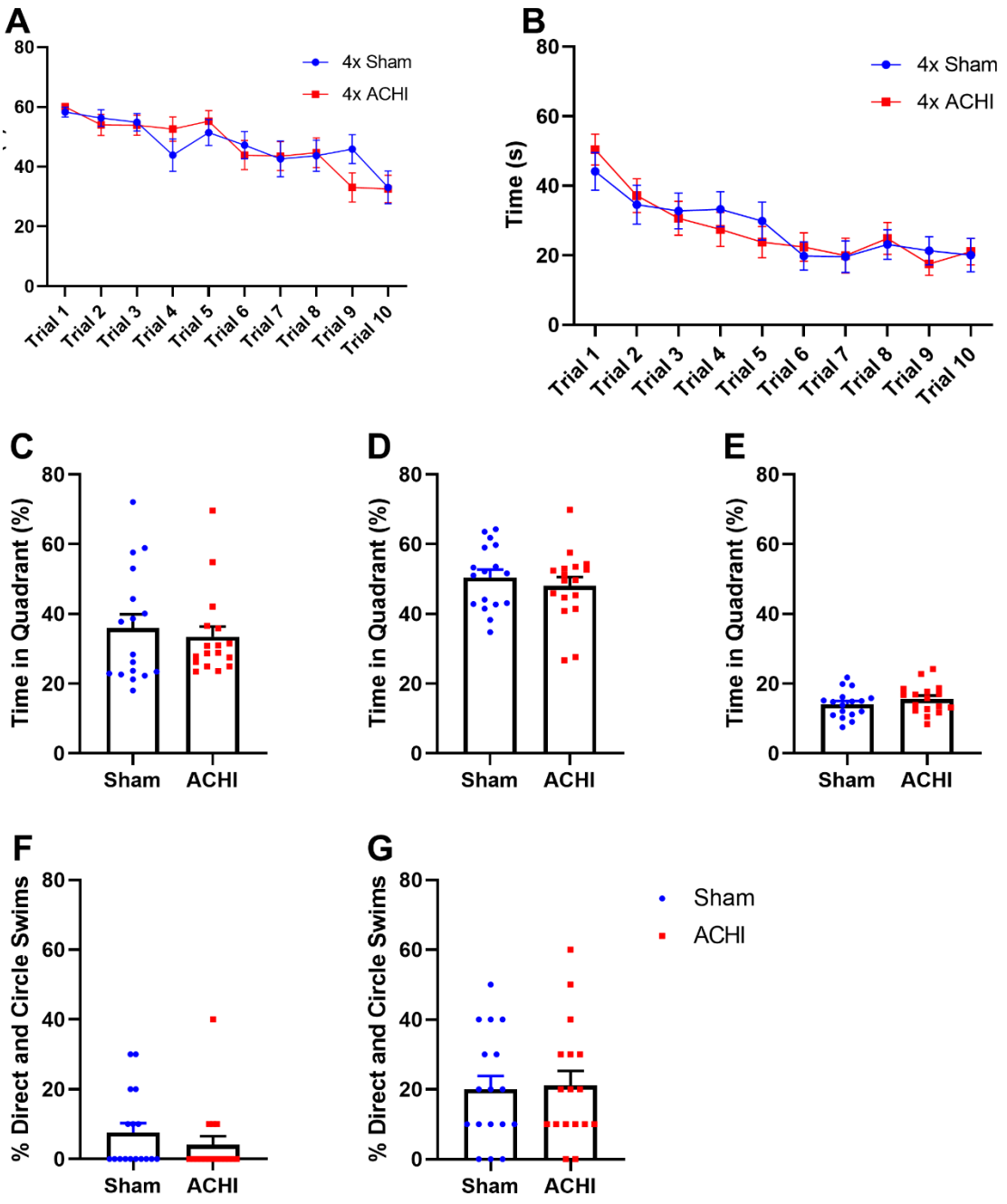

Figure. 3.6. Chronic effects of repeated ACHI on spatial working memory. No differences were observed at 3.5 months post-final sham/ACHI in **A)** latency to platform at acquisition or **B)** reversal between repeated sham and ACHI rats. n=17/group. Data presented as mean ± SEM. No differences were seen in **C)** time spent in target quadrant at acquisition, **D)** reversal and **E)** percentage of time spent in old target quadrant between repeated sham and ACHI rats at 3.5 months post-final sham/ACHI. No differences between repeated sham/ACHI rats were seen in **F)** direct or circle swims at acquisition and **G)** reversal. n=17/group. Data presented as mean + SEM.

3.3.5 Acute but not chronic elevation in serum NfL levels

A Simoa HD-X Analyser was used to quantify serum NfL levels at 7 days (n=8) and 3.5 months (n=8) after repeated sham or repeated ACHI (Figure. 3.7). Two-way ANOVA found a significant main effect of injury ($F_{(1, 27)}$=16.57; p<0.001), time ($F_{(1, 27)}$=6.40; p=0.018), and interaction of injury and time ($F_{(1, 27)}$=15.48; p<0.001) (Figure. 3.7A). *Post hoc* analysis revealed a significant difference in serum NfL between sham and ACHI rats at 7 days (p<0.0001) but not 3.5 months (p=0.994). A positive correlation (Pearson r=0.72; p=0.044) was found between 7-day serum NfL and the average number of beam slips immediately after each ACHI procedure (Figure. 3.7B). A positive correlation was also revealed between 7-day serum NfL and beam traverse time at three days post-injury (Spearman r=0.76; p=0.037) (Figure. 3.8E). No other correlations were observed for beam performance and serum NfL at seven days, 3 days or 3.5 months (Figure. 3.7C, D, F, G).

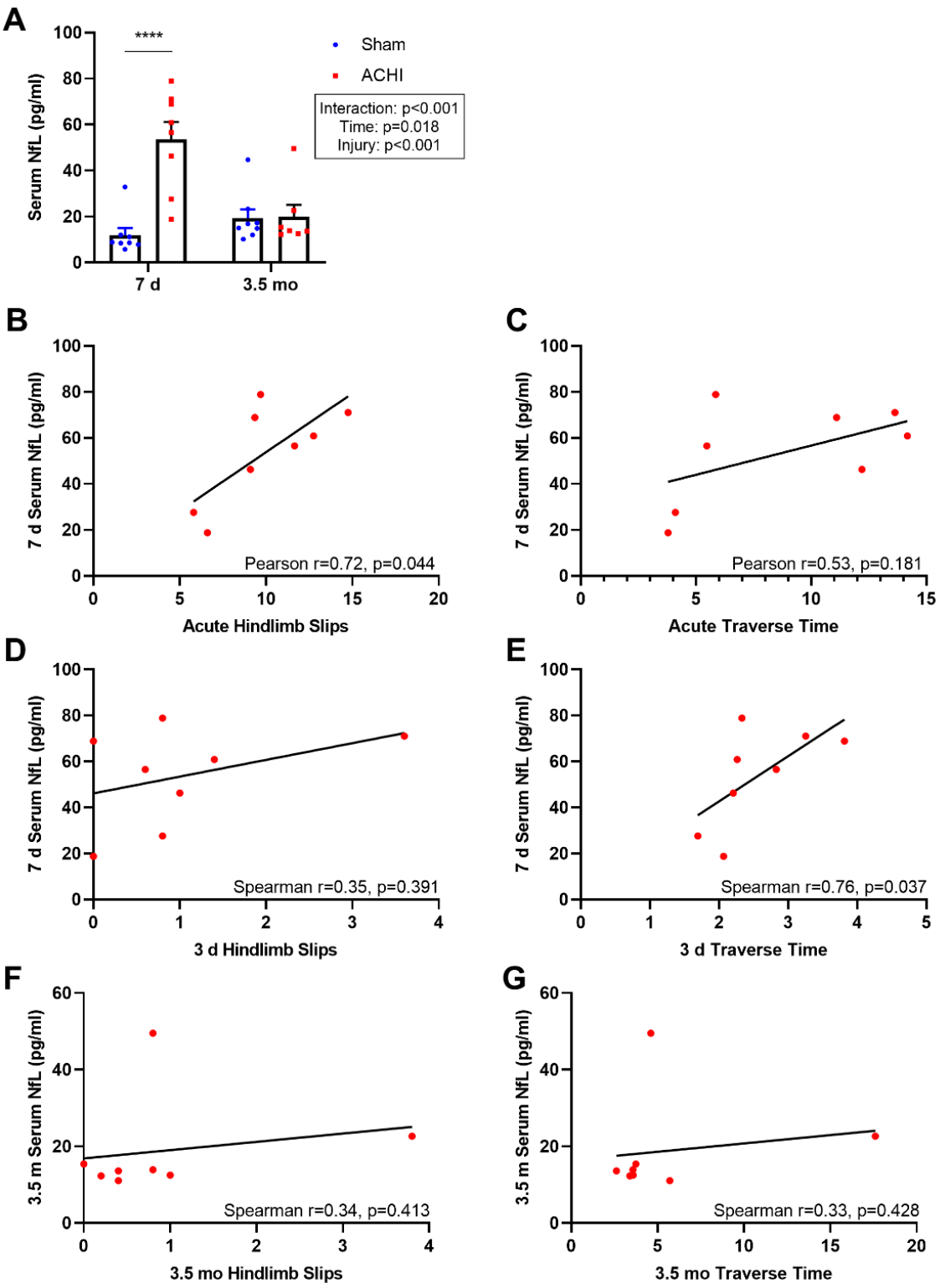

Figure. 3.7. Serum NfL levels after repeated ACHI. **A)** Serum NfL was significantly elevated at 7 days post-final ACHI (****p<0.0001) but not at 3.5 months when compared to repeated sham rats. n=8/group. Data presented as mean + SEM. **B)** A significant relationship between serum NfL levels at 7 days and average beam hindlimb slips immediately post ACHI at each timepoint (Pearson r=0.72, p=0.044) **C)** but did not correlate with average traverse time to immediately post-ACHI (Pearson r=0.53;

p=0.181). **D)** No correlation as found between NfL levels at 7 days and average hindlimb slips at 3 days (Spearman r=0.35; *p*=0.391), **E)** but was found with average beam traverse time 3 days post ACHI. Serum NfL levels at 3.5 months did correlate with **F)** beam slips (Spearman r=0.34; *p*=0.413) or **G)** traverse time (Spearman r=0.33; *p*=0.428) at this chronic time-point. A significant correlation Linear regression line of best fit is plotted for visualisation purposes **(B-G)**. n=8/group.

3.3.6 Widespread reductions in white matter FA

TBSS was used to assess FA of white matter tracts of *ex vivo* sham and repeated ACHI rat brains at 3.5 months (n=8/group). Widespread, significant reductions in white matter FA were observed in both contralateral and ipsilateral hemispheres (Figure. 3.8). No volumetric differences were seen in the ipsilateral and contralateral cortex, CC and hippocampus between *ex vivo* scans of 3.5 month repeated sham and mTBI brains (results not shown).

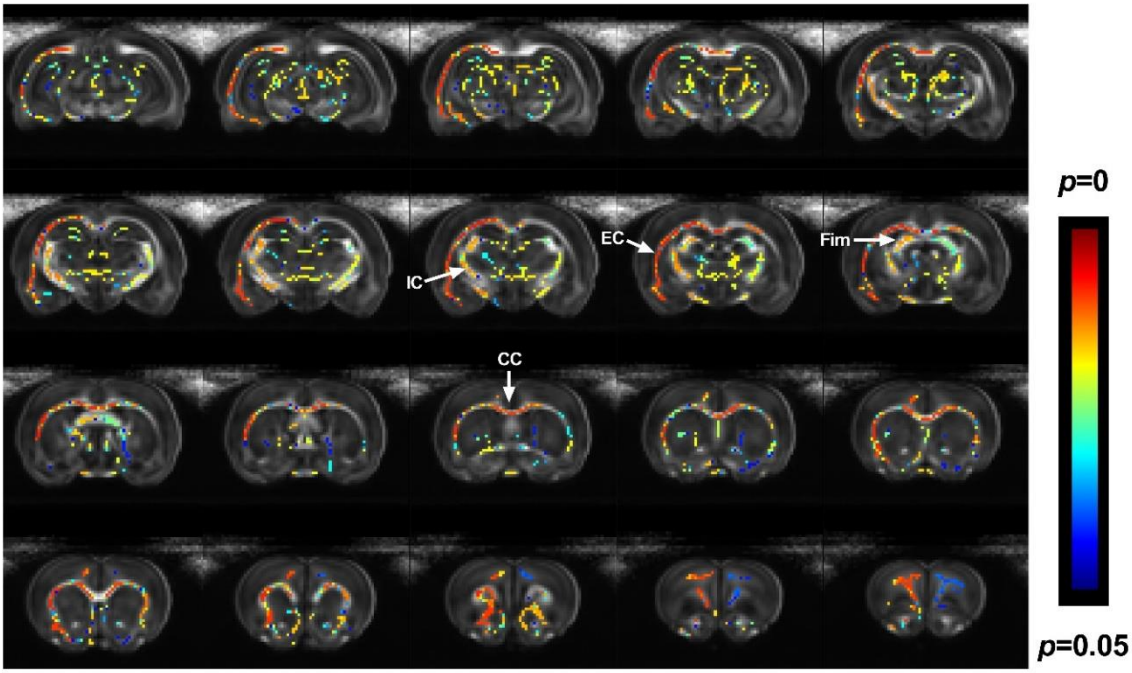

Figure. 3.8. Whole-brain TBSS revealed chronic FA changes in response to repeated ACHI. This figure depicts an anatomical overlay of the whole brain white matter skeleton of all sham rats versus all mTBI rats at 3.5 months post-injury, with coloured voxels representing significant group differences in FA for each voxel (as per key). When compared to sham rats, repeated ACHI rats had reduced FA in voxels of various matter tracts. In particular, reduced FA appeared most evident in the corpus callosum, external capsule, internal capsule, fimbria of hippocampus and corticofugal pathways (indicated by with white arrows). n=8/group. CC= corpus callosum, EC = external capsule, IC = internal capsule, Fim = fimbria of the hippocampus, Cf P = corticofugal pathways.

3.3.7 Acute and chronic alterations in the hippocampal proteome

High-resolution, data-independent acquisition mass spectrometry was used to analyse the proteomic differences between fresh ipsilateral hippocampal tissue (n=4-6/group) isolated from sham and repeated mTBI rats, and a total of 5053 proteins has been quantified across all samples. At seven days, 26 proteins were found to be significantly altered between sham and repeated mTBI rats (Figure. 3.9A), and 72 proteins were significantly different in expression at 3.5 months (Figure. 3.9B) when

considering a false discovery threshold of 0.05. String database analysis of GO terms revealed 78 biological functions altered at seven days, and 182 altered at 3.5 months (data not shown). After reviewing GO terms, the Uniprot database [263], and the literature, a selection of proteins deemed to have evidence for potential involvements in neurobiology and neuropathology were selected and presented in Table 2.

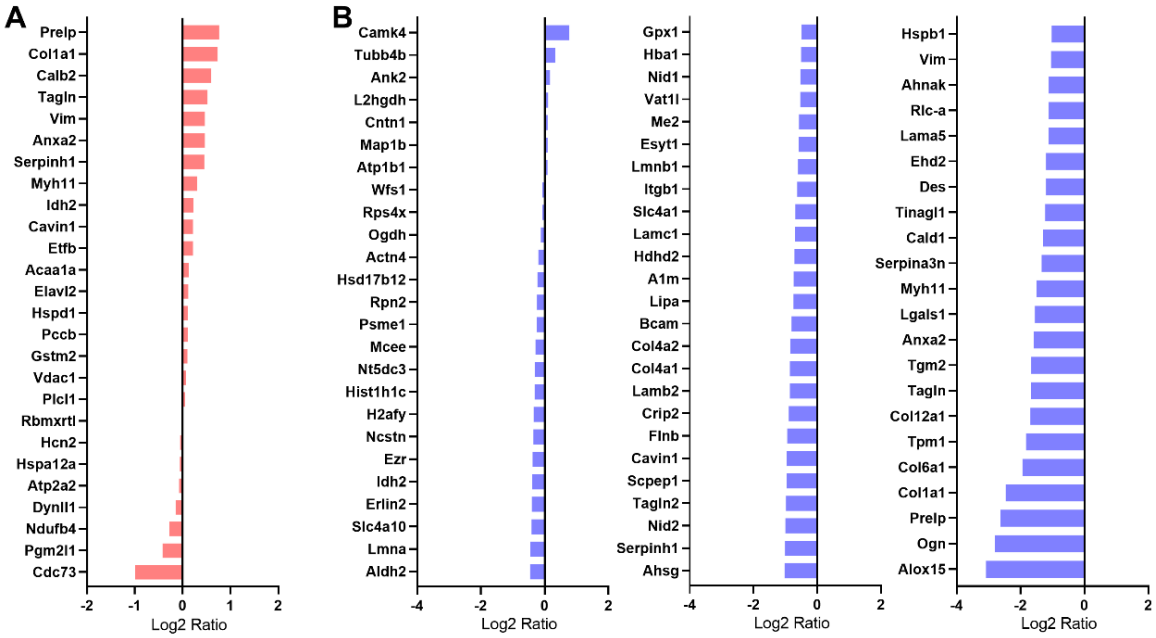

Figure. 3.9. Alterations in the hippocampal proteome. Proteins of the ipsilateral hippocampus of **A)** 7 day and **B)** 3.5 months post-repeated sham/ACHI with Q-value less than 0.05 revealed by mass spectroscopy. n=4-6/group. Data presented in log2 ratios.

Table. 3.1. Proteins of interest with altered ipsilateral hippocampal expression at 7-days following repeated mTBI compared to sham

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
3-ketoacyl-CoA thiolase, peroxisomal	↑	Acetate CoA-transferase activity	Mitochondrial fatty acid beta-oxidation	[264]
Annexin A2	↑↑	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	[265, 266]
Calretinin	↑↑↑	Ca^{2+} binding	Ca^{2+} buffering. Regulation of presynaptic cytosolic Ca^{2+} concentration and long-term potentiation	[267, 268]
Collagen alpha-1(I) chain	↑↑↑	Fibrillar forming collagen	Component of glial scar	[269, 270]
Dynein light chain 1, cytoplasmic	↓	Microtubule transport	Enables retrograde motility of vesicles and organelles along microtubules	[271]
ELAV-like protein 2	↑	RNA binding	Neurodevelopment and synaptic function.	[272, 273]
Electron transfer flavoprotein subunit beta	↑↑	Electron transfer activity	Mitochondrial fatty acid beta-oxidation and amino acid catabolism	[274]
60 kDa heat shock protein, mitochondrial	↑	Chaperonin involved in mitochondrial protein import	Mitochondrial protein import and macromolecular assembly. Promotes cytokine release and immune cell activation	[275, 276]
Isocitrate dehydrogenase [NADP], mitochondrial	↑↑	Isocitrate dehydrogenase ($NADP^+$) activity	Mitochondrial enzyme that plays an essential role in intermediary metabolism and energy production	[277, 278]
Myosin-11	↑↑	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	[279]

Propionyl-CoA carboxylase beta chain, mitochondrial	↑	Propionyl-CoA carboxylase activity	Mitochondrial catabolism of odd chain fatty acids, branched-chain amino acids and other metabolites	[280]
Vimentin	↑↑	Class-III intermediate filaments of non-epithelial cells	Marker of reactive astrocytes. Promotes elongation of astrocytic processes and glial scar formation	[281, 282]

Significantly altered hippocampal proteins at 7 days post- repeated ACHI. Proteins of interest with significant changes in the ipsilateral hippocampus at 7 days post final-ACHI relative to sham. Up regulation = #. Down regulation = $. Single arrow = 5-15%. Double arrows 15-50%. Triple arrows >50%. n=4-6/group.

Table. 3.2. Proteins of interest with altered ipsilateral hippocampal expression at 3.5 months following repeated mTBI relative to sham

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
Alpha-actinin-4	↓	F-actin crosslinking protein	Important for cell motility and structural plasticity of neurons	[283, 284]
Alpha-2-HS-glycoprotein	↓↓↓	Kinase inhibitor activity	Anti-inflammatory marker	[285, 286]
Arachidonate 15-lipoxygenase	↓↓↓	Non-heme iron-containing dioxygenase	Generates anti-inflammatory molecules in hippocampal-prefrontal cortex. Depletion may compromise cognition	[287]
Ankyrin-2	↑	Ion channel binding	Axonal domain organisation and establishment. Essential for synaptic stability. Adaptor and scaffold for various neuronal ion channels	[288, 289]
Annexin A2	↓↓↓	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	[265, 266]
Sodium/potassium-transporting ATPase subunit beta-1	↑	Regulatory hydrolysis of ATP	Preservation of Na^+/K^+ ATPases	[290, 291]
Non-muscle caldesmon	↓↓↓	Actin binding protein	Enhances axon extension in hippocampal neurons	[292]
Contactin-1	↑	Nervous system development	Present at junctions of axons, myelin, and glial cells. Role in myelin sheath development	[293]
Isocitrate dehydrogenase [NADP], mitochondrial	↓↓	Intermediary metabolism and energy production	Deficiency can cause oxidative stress and intensify mitochondrial dysfunction leading to neuronal death	[277, 278]
Integrin beta-1	↓↓↓	Collagen receptor	Integrin beta-1 signalling promotes axon assembly. Removal causes	[294, 295]

			axon formation deficits	
Galectin-1	↓↓↓	Lectin, binding carbohydrates	Neuroprotection in ischemic brain injury	[296, 297]
Lamin-B1	↓↓↓	DNA binding	Neuronal migration. Deficiency leads to abnormal neuronal development	[298]
Microtubule-associated protein 1B	↑	Microtubule binding	Present in axons and neurons. Essential for regulation of microtubule and synaptic interaction and stability	[299, 300]
Myosin-11	↓↓	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	[279]
2-oxoglutarate dehydrogenase, mitochondrial	↓	Oxoglutarate dehydrogenase (NAD+) Activity	Impairment of Ogdh complex has key role in glutamate mediated neurotoxicity during TBI, leading to neuronal death	[301]
Tubulin beta-4B chain	↑↑	Structural component of cytoskeleton	Major structural constituent of microtubules. Important to neurodevelopment and plasticity	[302]

Significant hippocampal proteome alterations 3.5 months after repeated ACHI. Proteins of interest with significant changes in the ipsilateral hippocampus at 3.5 months post final-ACHI relative to sham. Up regulation = #. Down regulation = $. Single arrow = 5-15%. Double arrows 15-50%. Triple arrows >50%. n=4-6/group.

3.4 Discussion

The neurobehavioural and pathophysiological alterations induced by repeated mTBI are still poorly understood. To provide new insights into the nature and progression of these changes, we implemented this clinically relevant rodent model of mTBI, along with detailed behavioural testing, blood and DTI biomarkers of axonal injury, and mass spectrometry of the hippocampal proteome. The current findings revealed that repeated ACHI was associated acutely with sensorimotor and subtle cognitive deficits, robust increases in serum NfL levels that were correlated with motor deficits, and increased levels of hippocampal proteins linked with changes in energy metabolism and glial activation. At the chronic stages there were subtle neurobehavioural deficits, widespread reductions in white matter integrity, and alterations in several hippocampal proteins including those associated with neurogenesis.

3.4.1 Behavioural deficits

Clinically, balance and gait are commonly assessed in acutely in patients suspected of having a mTBI [2], with deficits usually resolving within days [87]; however, there is some evidence that those with a history of mTBI may display prolonged subtle balance and gait deficits [303, 304]. Here, repeated mTBI resulted in significant sensorimotor deficits on the beam immediately after each impact, providing an indication of injury consistency. Analysis of beam performance at three days and 3.5 months revealed that repeated mTBI rats had sensorimotor impairments. **Chapter 2** found that a single ACHI induced beam deficits that resolved within 24 h, thus indicating that repeated ACHI appears to have prolonged the duration of sensorimotor deficits. A main effect of time was found for time to traverse the beam, likely reflective of the increased age or size of the rats at the chronic time-point [257].

Impairments to memory are also common in clinical mTBI [2], and repeated mTBI may cause cumulative and prolonged cognitive deficits [9, 305-307]. Y-maze testing revealed an injury by time interaction for relative time spent in the novel arm, providing some evidence of acutely impaired spatial memory in repeated mTBI rats. However, post-hoc analysis did not reveal a significant acute spatial memory impairment in repeatedly injured rats. There were no deficits Y-maze and water maze test results at the chronic time-point. Perhaps tests conducted over multiple sessions (e.g. Morris water maze) may be required to detect more robust visuospatial memory deficits at acute stages and chronic time-points post-injury. Nonetheless, the current findings indicate that in contrast to some other pre-clinical studies repeated mTBI did not result in overt and persisting spatial memory deficits [308]; however, differences in injury model, severity and schedule are likely to be contributing factors.

3.4.2 Axonal injury

TBSS: At 3.5 months, *ex vivo* MRI revealed no overt structural changes in all repeated mTBI rats, with volumes of the ipsilateral and contralateral cortex, CC and hippocampus all comparable to sham rats. Analyses of white matter integrity with TBSS did however reveal significant diffusion differences in repeated mTBI and sham rats, with reductions in FA observed in hemispheres ipsilateral and contralateral to the site of impact.

Reduced FA is commonly reported following mTBI in humans in both acute and chronic stages of injury [182, 247, 309, 310]. Although FA can also be increased by factors such as gliosis after TBI [311], reduced FA is widely thought to reflect white matter pathology such as axonal damage, demyelination, and reduced fibre density [311, 312]. Although the extent of FA reductions appeared to be greater in the contralateral hemisphere, widespread changes in white matter structures were observed throughout the

brain, with the CC, external capsule, internal capsule, and the fimbria of the hippocampus and corticofugal pathways particularly affected. Notably, these regions are commonly affected in clinical mTBI [182, 313-315], and we have previously found DTI changes in many of these regions in the acute stages after repeated ACHIs [255]. As such, damage to these regions may have been a key component of the cognitive deficits observed acutely, and the prolonged sensorimotor deficits. For example, the CC and corticofugal pathways are both thought to be critical to sensorimotor function, therefore damage to these regions may have contributed to the beam deficits in repeated mTBI rats [316, 317]. Nonetheless, further studies are required to understand the contribution of white matter changes to symptoms, as well as the temporal progression of these changes throughout acute, sub-acute stages after repeated mTBI.

Serum NfL: Quantification of serum NfL at seven days and 3.5 months after repeated mTBI allowed additional insights into the nature and progression of axonal damage. A cytoskeletal protein that is abundant in large-calibre myelinated axons, NfL is thought to be released into body fluids such as the cerebrospinal fluid [58, 59] and blood [318] following axonal injury or degeneration. Numerous studies have found serum NfL to be a dynamic biomarker of axonal pathology neurodegenerative diseases [319], with diffuse axonal injury hypothesised to be a key component of symptom outcomes following TBI [320, 321].

In the present study, we found a robust increase of NfL concentrations in serum at seven days after repeated ACHI compared to repeated sham rats. This result is similar to findings in studies of boxers exposed to multiple head traumas, with increased serum NfL found at 7-10 days after competition [60]. Despite excellent rodent cross-reactivity of the Simoa NfL assay, relatively few pre-clinical studies have utilised this assay in the context of TBI [251, 322]. This novel finding of robustly elevated NfL in serum at seven days

after repeated mTBI indicates significant potential of this assay in pre-clinical research, and further indicates that the extent of axonal damage was likely substantial in the ACHI model. Moreover, increases in serum NfL correlated with acute sensorimotor deficits provides evidence of the potential of this biomarker as an indicator of mTBI severity.

A plethora of recent studies have found that serum NfL is highly sensitive to axonal damage in neurodegenerative diseases [319]. Indeed, there is also emerging evidence that white matter damage may be a key contributing factor to some cases of PPCS [39, 323]. Given the associations with these conditions and repeated mTBI exposure, we hypothesised that serum NfL would remain elevated at 3.5 months post-injury; however, despite extensive white matter changes apparent at this stage, the resolution of this biomarker to sham levels is likely to indicate a lack of ongoing axonal damage. Future repeated mTBI studies are required to understand if neurodegeneration is triggered at later points (e.g. 6-12 months post-injury) that may correspond to the typical age of onset of neurodegenerative conditions in humans (i.e. >60 years of age), and whether serum NfL levels can predict and monitor the rate and extent of potential neurodegeneration.

3.4.3 Hippocampal proteome

A variety of notable differences were found in the hippocampal proteome at seven days or 3.5 months post-injury. Although requiring further investigation, these findings shed new light into the short- and long-term effects of repeated mTBI.

Acute alterations: Unbiased mass spectroscopy revealed up-regulation of several mitochondrial proteins, particularly those associated with β-oxidation of fatty acids. Although the energy demands of the brain are primarily met by Glc [324], recent studies have indicated that capacity of the brain to oxidise fatty acids is greater than previously

thought [325]. As such, given that significant reductions in cerebral Glc metabolism have been reported in the days after single and repeated mTBI [94], it is possible that increased fatty acid catabolism represents a compensatory mechanism in order to meet the metabolic requirements of the injured brain. Furthermore, given the high lipid concentrations in the brain, increased β-oxidation may also represent a protective mechanism to minimise a potentially toxic build-up of fatty acids released by cellular damage. Future studies are required to test these hypotheses, and to understand whether β-oxidation of fatty acids may represent a novel target for neuroprotective therapies and biomarkers of injury neurobiology.

In addition to the proteins associated with adaptations to hippocampal energy metabolism, we also observed increases in proteins associated with gliosis and the formation of a glial scar, buffering of Ca^{2+}, and maintenance of endothelial cell integrity. These findings provide further evidence that neuroinflammation, disrupted Ca^{2+} homeostasis, and cerebrovascular damage, are all likely prominent in the acute and sub-acute stages after repeated mTBI.

Chronic alterations: We observed some increases in protein expression at 3.5 months after repeated mTBI that may be reflective of neurorestorative functions activated by repeated mTBI. For example, there is evidence that ankyrin 2 and contactin-1 are critical to formation and organisation axons and synapses [289, 326, 327]. Contactin-1 also has a central role in oligodendrocyte myelin formation. Moreover, increases in microtubule-associated protein 1B and tubulin beta-4b, two key components of the axonal cytoskeleton, provide further evidence of potential activation of repair mechanisms after repeated mTBI.

In contrast, we also found a reduction in several proteins in animals subjected to repeated mTBIs that may be associated with impairments in neurodevelopment or

neuroremodelling, or a reduced neurogenic capacity. For example, we observed reductions in caldesmon and myosin II, with the interaction of these two proteins previously shown to be critical to axonal extension in hippocampal neurons [292]. In addition, integrin beta-1 is a cell surface receptor thought to have diverse and important roles in the central nervous system [328], including outgrowth of hippocampal progenitor cells [329] and axon assembly during development [294], with multiple studies demonstrating that deficiency of integrin beta-1 results in impaired long-term potentiation [295], synaptic plasticity and working memory [330]. Although further research is required to confirm and understand the significance of these decreases in protein expression, when considering the significant literature on their neurobiological importance, we speculate that these proteomic changes may contribute to chronic neuropathology and behavioural impairments after repeated mTBI.

Although this study has provided some important new insights into the potential effects of repeated mTBIs, there are some limitations to the study that should be acknowledged. Firstly, it is important to note that given this is a single sex study, there may be limitations in generalising the current findings to females. Second, although we chose not to run acute behavioural analysis on the chronic recovery rats to avoid the potential confound of repeated task exposure, serial testing may have provided greater insights into the temporal progression of behavioural phenotypes after repeated mTBI. In addition, TBSS analysis was chosen to enable unbiased assessment of white matter throughout the brain; however, one limitation of this approach is that it does not enable correlation of FA values within a specific brain region with other variables such as serum NfL and sensorimotor deficits. However, serum NfL levels appear to normalise by the chronic stages of injury, and as such, we hypothesise that correlation with FA values appears unlikely. Although we observed robust changes in white matter with TBSS, further studies are required to validate these changes with complementary techniques such

as histology. Finally, proteomic analysis in this study was limited to the ipsilateral hippocampus and analysis with mass spectrometry, therefore further studies are required to validate these changes and investigate potential alterations in other brain regions.

3.5 Conclusions

With increasing concern that exposure to repeated mTBIs can lead to lingering or progressive neurological impairments, it is imperative that pre-clinical research is conducted to better understand these associations and their pathophysiological underpinnings. Using a clinically relevant rat model of repeated mTBI, the current acute recovery studies revealed that while still in the symptomatic period, rats had changes in several hippocampal proteins suggestive of adaptations in energy metabolism and increased glial reactivity, as well as a profound increase in serum axonal injury biomarker NfL that correlated with sensorimotor deficits. At a chronic stage of injury, despite displaying minimal neurobehavioural deficits and a return of NfL to sham levels, we found significant disruptions to the hippocampal proteome, and widespread reductions in white matter integrity. Given the significant promise of serum NfL as an early indicator of neurodegenerative disease onset, we speculate that resolution of this biomarker indicates that changes in white matter were not progressive at 3.5 months post-injury, with future studies required to determine if progressive neurodegenerative changes are apparent at a more chronic time-points.

Chapter 4: Acute effects of single and repeated mTBI on neurometabolite levels and mitochondrial function in rats

4.1 Introduction

mTBI, or concussion, is the most common form of TBI, with a 10-fold higher burden on healthcare than both moderate and severe TBI [3], making it a serious medical and societal issue [2]. mTBI is a heterogenous injury resulting in a large variety of neurobehavioural and motor consequences [2, 6]. Although still poorly understood, mTBI symptoms are thought to be caused by an acute and transient neuropathological cascade [331], with multiple mTBIs in short succession found to exacerbate and prolong symptoms and neuropathology [7, 148]. Furthermore, mounting evidence suggests that repeated mTBIs may lead to progressive neurodegenerative conditions including AD, ALS and CTE [10, 161, 243]. However, despite growing preclinical and clinical literature on the effects of repeated mTBI, much is still unknown about the cellular consequences of single and repeated injury.

One recognised and potentially prominent neuropathological aspect of the mTBI cascade is neurometabolic dysfunction [99, 188]. *In vivo* ^{1}H-MRS can identify the absolute concentrations of up to 20 metabolites, with alterations of these concentrations giving an indication of metabolic status in the brain. A number of these metabolites are postulated to have utility in diagnosing potentially concussed individuals and predicting patient outcome (Table 4.1).

Table. 4.1. Metabolites of interest: Summary of literature reporting metabolite alterations after clinical and/or preclinical TBI.

Metabolite	Interpreted Biological Function	TBI Effect	Ref
Glu	Excitatory neurotransmitter; stimulate glycolysis	↑	[332-334]
Gln	Storage form of Glu located in glia; aerobic glycolysis	↑ or ↓	[332-335]
Glc	Metabolised for ATP via glycolysis in the cytosol; anaerobic metabolism	**?**	[336]
NAA	Synthesised by mitochondria; marker of neuronal and axonal integrity	↓	[24, 253, 337, 338]
Cr+PCr	Marker of mitochondrial energy metabolism	↑ or ↓	[33, 36, 339]
GPC+PCh	Precursor of acetylcholine; marker of membrane damage	↑	[332, 338]

Glutamate, Glu; glutamine, Gln; glucose, Glc; N-acetylasparate, NAA; creatine and phosphocreatine, Cr+PCr; and glycerolphosphocholine and phosphocholine, GPC+PCh; adenosine triphosphate, ATP

One such neurometabolite, NAA (as described in **Chapter 1**), has been found to be a biomarker of neuronal integrity [24] and therefore is thought to be a useful marker in diagnosing mTBI. A clinical study observed temporal metabolite alterations post-concussion, with a second concussion in close succession found to prolong recovery and worsen depletion of NAA/Cr [188]. Notably, these metabolite alterations persisted beyond symptom resolution. Moreover, the same authors used complimentary rodent studies and found repeated mTBIs could lead to cumulative metabolic dysfunction, but only when injuries were separated by intervals of less than 5 days [95]. In addition, Cr and phosphocreatine (PCr) are metabolites abundant in metabolically active organs that need frequent surges of energy, such as the brain [29]. As such, they are thought to be an indicator of cellular energetics [340]. Whilst Cr concentration is thought to remain unchanged in the diseased brain [34], some studies have found TBI to significantly alter

Cr levels [33, 36, 339]. Therefore, the current study looked at potential changes in Cr+PCr using a clinically relevant rodent model of mTBI. Choline is a major component of phospholipid membrane and as such, the elevation of glycerolphosphocholine and phosphocholine (GPC+PCh) after TBI are thought to be a marker of cell membrane breakdown and therefore cell damage [332] and therefore were of interest in this study.

As mentioned in **Chapter 1**, TBI can trigger the uncontrolled release of Glu, leading to excitotoxicity and potentially cell death [92, 93]. Glutamine (Gln) is the storage form of Glu [333, 341]. Whilst previous literature does report on Glu and Gln alterations, difficulty in distinguishing their peaks on early ^{1}H-MRS spectra led to many previous studies to report Glu and Gln as a sum [33, 332, 342]. Updated technology available for the current study allowed for these two metabolites to be reported separately, and as such, provided an opportunity to add to the limited literature of these metabolites in the ^{1}H-MRS. Lastly, as briefly described in **Chapter 1**, the effect of TBI on Glc metabolism leading to hyper-glycolysis [95] has been postulated. However, to the best of my knowledge, Glc concentration alterations after mTBI has not been reported in rodent ^{1}H-MRS studies.

Altogether, it has been speculated that metabolic changes due to mTBI might be responsible for a temporary period of ICV, with shorter inter-injury intervals leading to increased neurometabolic deficits [95, 188]. However, the nature and mechanisms of metabolic changes after single and repeated mTBI remain poorly understood.

Mitochondria are vital in regulating cellular metabolism, by producing ATP and buffering intracellular Ca^{2+}; however, these functions can be disrupted following mTBI. Studies have shown moderate and severe TBI to increase Ca^{2+} influx, thus overloading mitochondria and resulting in ionic imbalances, loss of membrane potential and impairment of ATP production [343, 344]. Furthermore, mitochondrial dysfunction after

TBI in rodents can exacerbate or contribute to accumulation of ROS resulting in oxidative damage [108]. Importantly, a high percentage of significantly altered hippocampal proteins in **Chapter 3** (i.e. seven days after repeated mTBIs) were related to mitochondrial energy metabolism. This provided evidence that repeated ACHI produced metabolic changes, warranting further investigation into the exact mechanisms by which these changes occur.

Given mitochondrial dysfunction seems to heavily influence neurometabolism after TBI, and the ability that ^{1}H-MRS has to detect neurometabolic changes *in vivo*, it is surprising that few preclinical studies have investigated the effect of TBI on metabolite alterations detected by ^{1}H-MRS. Therefore, this chapter aimed to investigate potential metabolic changes after single and repeated mTBI using *in vivo* ^{1}H-MRS, as well as the underlying pathological mechanisms associated with these potential changes. By using the novel surgery- and anaesthetic-free ACHI rat model, findings in this study could potentially uncover translatable mTBI biomarkers, improve patient management and reveal therapeutic targets. A single mTBI was hypothesised to induce metabolite changes in ^{1}H-MRS that would be associated with mitochondrial deficits, and that these changes would be exacerbated after repeated injury at 24 h.

4.2 Materials and Methods

4.2.1 Animals

Forty-eight male Sprague Dawley rats were obtained from Monash Animal Research Platform (Clayton, Victoria, Australia) at PND 25-27. Three rats were housed per cage with a 12h:12h light/dark cycle and food and water available *ad libitum*. All procedures were performed in accordance with the Alfred Medical Research and Educational Precinct Animal Ethics Committee at Institute (E/1832/2019/M) and were

within the ARRIVE guidelines and the Australian code of practice for the use and care of animals for scientific purposes by the Australian National Health and Medical Research Council. All animals were given two days to acclimatise to their enclosures upon arrival before experimenter contact. After this period animals were handled four days per week until the first sham or injury procedure on PND 38-41.

4.2.2 Mild TBI

Rats were randomly allocated into groups that received five sham injuries, four sham injuries followed by an ACHI (i.e. a single ACHi group), or five ACHI procedures, with each procedure separated by one day. Sham and ACHI procedures were completed similar to those described previously in **Chapters 2** and **3**. In brief, a steel helmet was placed over the rat restrained via a Decapicone™ (Braintree Scientific, MA, United States), with the impact site centered over the left parietal bone. The restrained rat was then placed on a foam bed and held in position by body. Once positioned, a 5 mm tip attached to a controlled cortical impactor (Leica Biosystems, IL, United States) was triggered to strike the helmet (velocity: 6.5 m/s, depth: 4 mm, dwell time: 100 ms). The total duration of the procedure was less than 1 minute. Sham procedures were performed identically to ACHIs, with the impact triggered adjacent to the animal's head. No skull fractures were observed during post-mortem analysis. The lack of anaesthesia allowed for inspection of visual signs that may be associated with LOC (e.g. apnea, absence of hindlimb withdrawal reflex, lying motionless). Once rats had self-righted and were mobile (typically within 1-minute post-injury), the beam task was used to confirm injury for all rats immediately after each procedure (as described below). Three rats were excluded due to machine error during the mTBI procedure.

4.2.3 Video observation protocol

In **Chapters 2** and **3**, rats performed the beam task immediately after impact to provide an indication of injury consistency. However, to avoid a potential learning effect, the current study developed and incorporated a new scoring method for video signs of mTBI based on the recent injury definitions provided by the international consensus of video signs of concussion in professional sports [345]. A video observation protocol for signs of mTBI in rats was used to confirm injury after each procedure (Table. 4.2). The protocol consisted of 5 video signs of injury with a point given for each injury effect present. After completion of all experiments, video signs were scored by an experimenter blinded to the treatment of groups. Individual scores for each rat were calculated for analysis and used as a clinically translatable confirmation of injury.

Table. 4.2. Video observation protocol using video signs of mTBI in rats immediately post-impact[a]

Video signs	***Description***	***Score***[b]
Impact seizure/tonic limb/s	Visible signs of seizure or tonic tail/body posture	0/1
Lying motionless	Lying motionless for >2 sec	0/1
No protective action	Tail raise - Limb extension reflex impaired (splayed/folded in body)	0/1
Floppy	Body sprawled with no limbs on beam	0/1
Impaired motor coordination	>5 hindlimb slips in single trial beam task	0/1
Total score		**of 5**

[a]Adapted from the final international consensus definitions of video signs of possible sports-related concussion [345].

[b]Point given when a video sign of injury was observed

4.2.4 Beam task

Sensorimotor function was assessed via the beam task at baseline (1 day prior to the first injury), as well as at 1- and 4 hours post-final injury. The task consisted of a 2 cm-wide, 1.5 m-long wooden beam, elevated 75 cm above the ground, with a protective mattress placed below to soften any falls. At the start-end of the beam there was a bright light (i.e. an aversive stimulus), as well as a camera to record the task. At the finish-end, a dark "home box" was placed with home cage bedding to encourage completion of the task. Prior to testing, beam training took place as described in **Chapters 2** and **3**. Rats were required to traverse 0.5 metres on the beam to the home box to complete a trial, with five trials used for each session. In a small number of cases, trials were re-performed if rats were immobile for longer than five sec. Rats that failed to complete a trial within 20 seconds were assigned this value. Rats that fell were automatically assigned this maximum time as well as two hindlimb slips. Average latency to cross the beam and hindlimb slips were later determined from captured video analysis by a researcher blinded to the experimental conditions.

4.2.5 ^{1}H-MRS

Twenty-four hours after final injury, *in vivo* ^{1}H-MRS was performed using a 9.4 T Bruker MRI (Bruker™ BioSpin®, USA). Rats were anaesthetised in an enclosed clear plastic box with 5% isoflurane inhalation in 100% oxygen at a flow rate of 2 L/min. After rats lost consciousness, they were carefully positioned supinely in a specially built rat cradle, with their head fixed and a nose cone placed over their snout to deliver approximately 2% isoflurane in 100% oxygen at a flow rate of 500 mL/min to maintain anaesthesia throughout the ~ 50 – 60 min procedure. Body temperature was maintained with a heating pad connected to a water heating circulation system built into the rat cradle.

Point resolved spectroscopy (PRESS) was acquired in the ipsilateral and contralateral hippocampus with VAPOR water suppression and outer volume saturation. An actively-decoupled, cryogenically-cooled, surface coil was used together with a volume resonator for excitation. Acquisition parameters were: repetition time = 2500 ms; echo time = 16.5 ms; number of excitations = 256; voxel size = 2.5 × 1.5 × 2.4 mm^3; and 4096 points over 11 ppm. A non-water suppressed spectra was also acquired for absolute quantification of metabolite concentrations relative to water. LCModel was used to process ^{1}H-MRS data with a metabolite basis set matched to field strength and echo time.

As described in Chapter 1, Cr and/or phosphocreatine (PCr) is commonly used as a denominator to report metabolite quantification in ratios. However, evidence suggests that Cr and PCr may be altered following brain injury [33, 36, 339]. As such, the current study used absolute concentrations to quantify Glu, Gln, Glc, NAA, Cr+PCr and GPC+PCh (Table 4.1).

4.2.6 Mitochondrial Isolation for Metabolic Flux Assay

Seahorse XF96 Extracellular Flux Analyser (Agilent, Santa Clara, CA, United States) was used to measure the effect of single and repeated mTBI on the oxygen consumption rate (OCR) of isolated mitochondria. At 24- (no ^{1}H-MRS) or 30-hours (^{1}H-MRS) after final injury, rats were anaesthetised via isoflurane inhalation, before being injected intraperitoneally with Lethobarb (80 mg/kg) and decapitated. The rostral half of the ipsilateral hippocampus was dissected from each rat and immediately placed in ice-cold buffer A (220 mM Mannitol, 70 mM Sucrose, 20 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES), 2 mM Tris-hydrochloride (Tris-HCl), 1 mM Ethylenediaminetetraacetic acid/Ethylene glycol tetraacetic acid (EDTA/EGTA), 0.4%w/v bovine serum albumin, pH 7.2) and homogenised using a Dounce homogeniser. Contents were then pipetted into an Eppendorf tube and kept on ice until all rats were culled (n=6-12/cohort). The homogenate was then centrifuged at 650 x g for 5 min, the

pellet was discarded, and the supernatant retained and transferred to a fresh tube. The supernatant was centrifuged again at 650 x g for 5 minutes and any pelleted material was removed with a long thin pipette. The resultant supernatant was then transferred to a high-speed centrifuge tube and centrifuged at 10,000 x g for 5 min, the supernatant was discarded, and the mitochondrial pellet was resuspended in Isolation buffer A. Mitochondria were re-pelleted by centrifuging at 10,000 x g for 5 min, discarding the supernatant and resuspending the pellet in 1 ml Isolation buffer B (220 mM Mannitol, 70 mM Sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2). The final pellet was collected by centrifuging at 10,000 x g for 5 min, discarding the supernatant and resuspending the pellet in Isolation buffer B at a ratio of 1.5 ml per 1 g of original tissue weight. A BCA (BioRad) protein assay was performed and mitochondria were diluted in Mitochondrial Assay Solution (MAS) buffer (sucrose 70 mmol/L, mannitol 220 mmol/L, potassium phosphate monobasic 5 mmol/L, magnesium chloride 5 mmol/L, HEPES 2 mmol/L, EGTA 1 mmol/L, 10 mM Malate, 10mM pyruvate, bovine serum albumin fatty acid free 0.2%, pH 7.4) and plated in a 96-well Seahorse V3-PS plate at 3 ug/well in replicates of 6 for dual assays and centrifuged at 2000 g for 20 minutes at 4°C to attach the mitochondria to the bottom of the plate. Mitochondria were then equilibrated in respective MAS buffer (180 µl/well) and incubated at 37°C in the absence of CO_2 for 10 min. The sensor cartridge was hydrated and kept at 38 °C overnight before analysis, in accordance with the Seahorse XF96 Extracellular Flux Analyser instructions.

The coupling and electron flow (ETF) assay were conducted simultaneously and consisted of repeat cycles of 3 minutes of waiting, and 4 minutes of measuring. 3 x initial measurements were taken for the coupling assay in MAS and for the ETF in MAS + 4 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP).

The coupling assay consisted of a base OCR measurement (State 2) before sequential injections of the following compounds and subsequent measurements of OCR

after each: ADP (3 mM) to initiate State 3, the ATP synthase inhibitor oligomycin (1 µM) to induce State 4 oligomycin (State 4o), the proton ionophore FCCP (1 µM) to initiate State 3 uncoupled (State 3u) and the mitochondrial complex III inhibitor antimycin-A (1 µM) to induce State 5 antimycin-A. The ETF assay consisted of sequential injections of the following compounds and subsequent measurements of OCR after each: the mitochondrial complex I inhibitor rotenone (2 µM), complex II substrate succinate (10 mM), antimycin-A (4 µM), and complex IV substrate L-Ascorbate (10mM)/N,N,N′,N′-Tetramethyl-p-phenylenediamine (100 µM). Finally, the respiratory control ratio (RCR) was calculated (State 3/State 4o) as a general measure of mitochondrial function.

4.2.7 Statistical Analysis

GraphPad Prism version 8.3.1 for Windows (GraphPad Software, CA, United States) was used to analyse all outcomes. ETF and coupling assays were analysed using a two-way ANOVA and are presented as mean ± standard error of the mean. RCR was analysed using an ordinary one-way ANOVA and is presented as mean ± standard error of the mean. All other data were analysed with two-way repeated measures ANOVA and are presented as mean ± standard error of the mean. Tukey's multiple comparisons was performed when appropriate. Statistical significance was accepted if $p<0.05$. Due to potential violations of assumptions of normality for beam data, additional analysis was performed for beam hindlimb slips with Friedman tests (corrected for multiple comparisons, alpha 0.0758). Beam traverse time data was transformed before analysed with a two-way ANOVA for baseline, 1 h and 4 h post-final ACHI.

4.3 Results

4.3.1 Video observation protocol confirmed injuries

A video observation protocol was used to confirm injury in rats immediately after each procedure (n=13-16/group; Figure. 4.1). Video signs for impact seizure/tonic posture or tail, lying motionless, absence of protective action, floppiness and impaired motor coordination were observed immediately after ACHI induced video signs of mTBI using the video observation protocol.

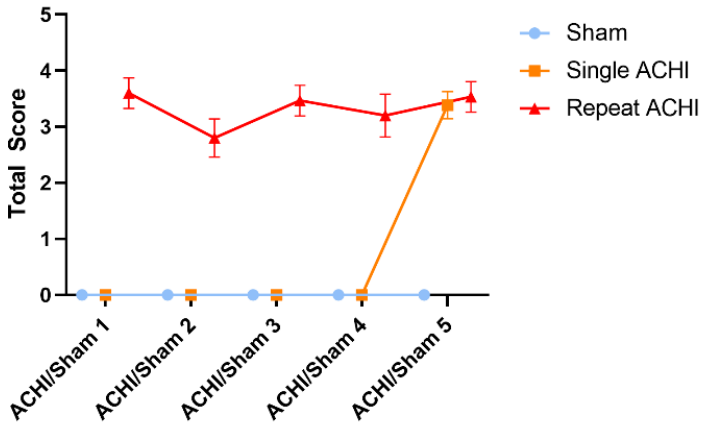

Figure. 4.1. Video observation protocol confirmed injury in rats immediately after each ACHI. Sham n=16 baseline, single ACHI n=13, repeated ACHI n=15.

4.3.2 Repeated ACHI induces acute sensorimotor deficits

Sensorimotor function was assessed using the beam task at baseline, 1- and 4-hours post-final injury (n=13-16/group; Figure. 4.2). Total number of hindlimb slips (Figure. 4.2A) were analysed using a two-way repeated measures ANOVA, revealing a main effect of injury ($F_{(2,41)}$=15.84, p<0.0001), time ($F_{(1.59,65)}$=10.34, p=0.0004) and significant injury by time interaction ($F_{(4,82)}$=9.75, p<0.0001). *Post hoc* analysis revealed that repeated ACHI rats displayed more hindlimb slips than sham (p=0.0059) and single injury (p=0.0098) rats at 1-hour post- final injury and maintained deficits for 4 hours post-final ACHI compared to sham (p = 0.026) and single ACHI (p=0.028). Average time

taken to traverse the beam (Figure. 4.2B) was analysed with a two-way repeated measures ANOVA, and found a main effect of injury ($F_{(2,41)}$=14.60, p<0.0001), time ($F_{(1.29,52.74)}$=6.40, p<0.0092) and interaction of time and injury ($F_{(4,82)}$=11.69, p<0.0001). *Post hoc* analysis found that repeated ACHI rats took significantly longer to traverse the beam at 1 hour post final ACHI compared to sham (p=0.0195) and single ACHI (p=0.0114) rats. At 4 hours post final injury, repeatedly injured rats still had significant sensorimotor deficits compared to sham (p=0.0009) and single ACHI (p=0.0018) rats.

Additional analyses was performed due to potential violations of assumptions of normality for beam data analysed with linear models (data not shown). For beam slips, a Friedman tests was used to compare beams slips at baseline, 1 h and 4 h within rats in each group, with alpha set to 0.017 due to correct for multiple comparisons. No differences were observed over time for sham (p=0.758) and single injury rats (p=0.452); however, a significant difference was found over time within repeat injury rats (p=0.003), with post-hoc test revealing a difference between baseline and 1 h (p=0.0052) and 4 h values (p=0.012). For traverse time, values were Ln transformed and analysed with two-way ANOVA, with a main effect of injury ($F_{(2, 41)}$=15.04, p<0.0001), no effect of time ($F_{(1.735, 71.12)}$=1.437, p=0.244), and an injury by time interaction ($F_{(4, 82)}$= 0.82, p<0.0001). Dunnett's multiple comparisons within each injury group revealed no difference between baseline and 1 h (p=0.071) and a significant improvement in sham animals from baseline to 4 h (p=0.046), no change from baseline in single injury rats at 1 h (p=0.137) and 4 h (p=0.6807), and within repeat injured rats, no difference from baseline at 1 h (p=0.060) but a significant decrease in performance compared to baseline at 4 h (p=0.031).

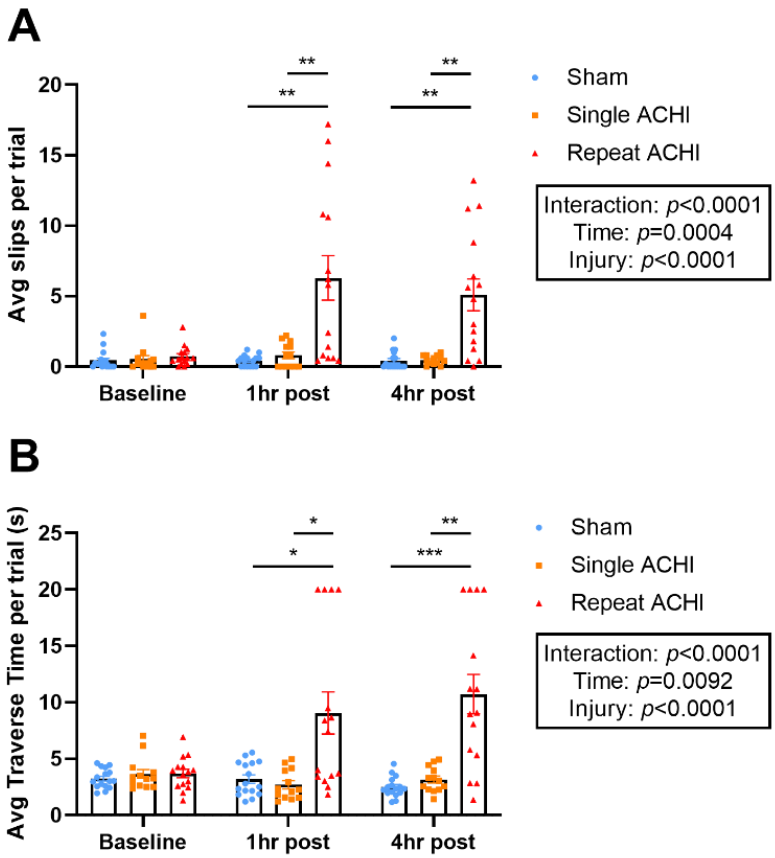

Figure. 4.2. Effect of single and repeated injury at baseline, 1- and 4 hours post-final mTBI. **A)** At 1- and 4 hours post-final ACHI, repeated ACHI rats displayed significantly more hindlimb slips than sham (1 hour and 4 hours; $p<0.01$) or single ACHI (1 hour and 4 hours; $p<0.01$) **B)** Repeated ACHI also took longer to traverse the beam than sham (1 hour, $p<0.05$; 4 hours; $p<0.01$) or single ACHI (1 hour, $p<0.01$; 4 hours; $p<0.001$) rats. Sham n=16 baseline, single ACHI n=13, Repeated ACHI n=15. Data are presented as mean ± SEM.

4.3.3 Repeated ACHI acutely altered some ^{1}H-MRS metabolites

^{1}H-MRS was used to detect metabolite alterations in the hippocampus at 24 hours post-final injury (n=9-12/group; Figure. 4.3). Gln concentrations were analysed using a two-way ANOVA and revealed a main effect of injury ($F_{(2,58)}$=3.813, p=0.0278), however no main effect of hemisphere or interaction of injury by hemisphere was found. Significant increase between sham and repeated ACHI groups (p=0.035) was revealed after *post hoc* analysis (Figure. 4.3A). Glc concentrations were assessed using a two-way ANOVA and found no injury by hemisphere interaction or main effect of hemisphere, but did reveal a main effect of injury ($F_{(2,56)}$=3.672, p=0.0317). *Post hoc* analysis revealed a significant decrease in Glc between sham and repeated ACHI (p=0.029; Figure. 4.3B). Lastly, two-way ANOVA analyses found no significant alterations in Glu, NAA, Cr+PCr and GPC+PCh concentrations between all groups (Figure. 4.3 C-F).

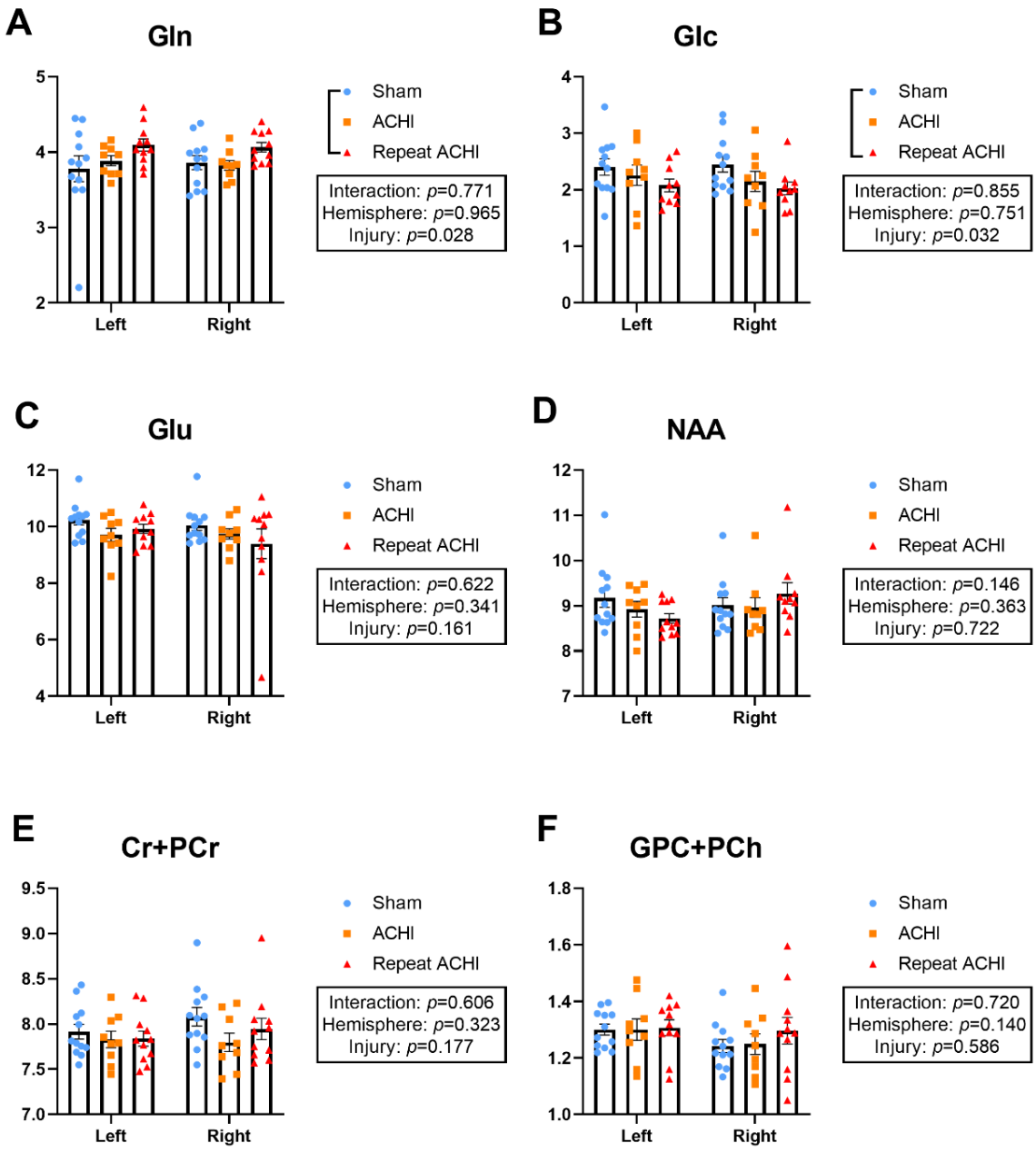

Figure. 4.3. Neurometabolic alterations at 24 hours after single and repeated mTBI. Gln was significantly elevated in repeated ACHI rats compared to sham rats (p=0.035). A significant decrease in Glc was revealed in repeated ACHI rats compared to sham (p=0.029). No significant alterations were observed in NAA< Cr+PCr and GPC+PCh between all groups. Sham n=12, single ACHI n=9, repeated ACHI n=11. Data are presented as mean ± SEM.

4.3.4 Hippocampal mitochondrial bioenergetics after single and repeated ACHI

Hippocampal mitochondrial bioenergetics were assessed after ^{1}H-MRS at 30 hours (Figure. 4.4; n=4-6/group) or without ^{1}H-MRS at 24 hours (Figure. 4.5; n=4-6/group) post-final procedure. Unavoidable variables associated with *in vivo* ^{1}H-MRS likely affected outcomes in this analysis (i.e. combining multiple cohorts of rats for analysis and varied resting times after scans prior to tissue collection). No group differences were found at 30 hours in the ETF assay (Figure. 4.4A), coupling assay (Figure. 4.4B) or RCR (Figure. 4.4C) of rats that underwent ^{1}H-MRS. At 24 h, more consistent results were produced from rats that did not undergo no ^{1}H-MRS, however no differences in the ETF assay (Figure. 4.5A), coupling assay (Figure. 4.5B) or RCR (Figure. 4.5C) were found.

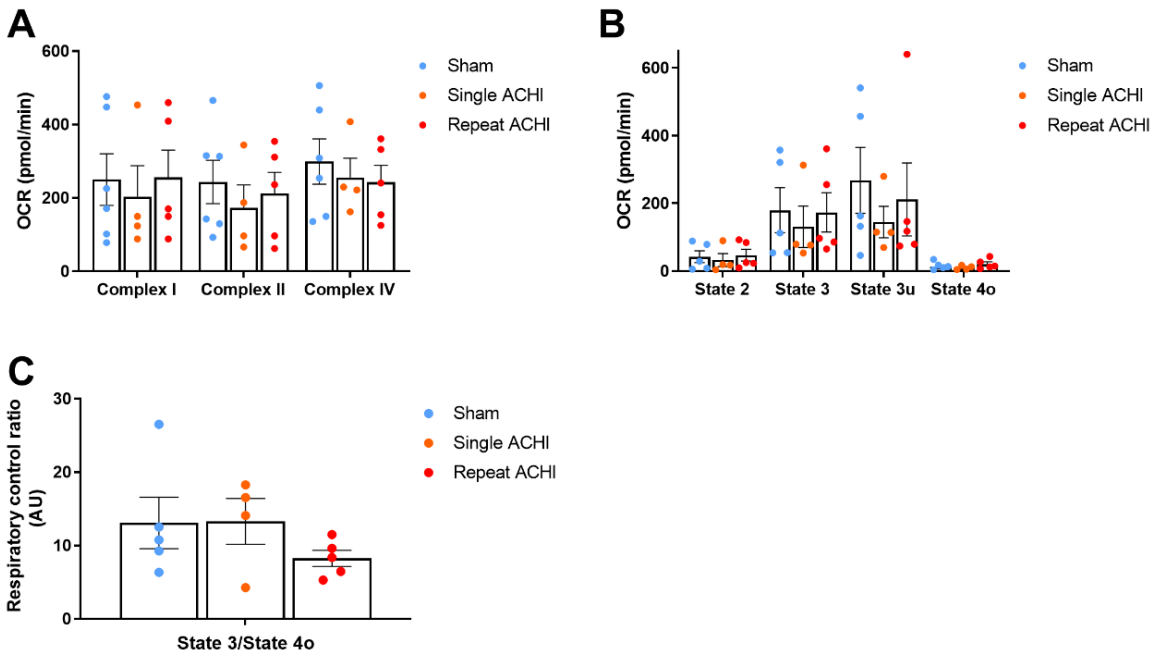

Figure. 4.4. Effect of single and repeated mTBI on isolated hippocampal mitochondrial respiration at 30 hours after ^{1}H-MRS. No differences were observed in the **A)** ETF assay, **B)** coupling assay or **C)** RCR. Sham n=6, single ACHI n=4, repeated ACHI n=5. Data are presented as mean ± SEM.

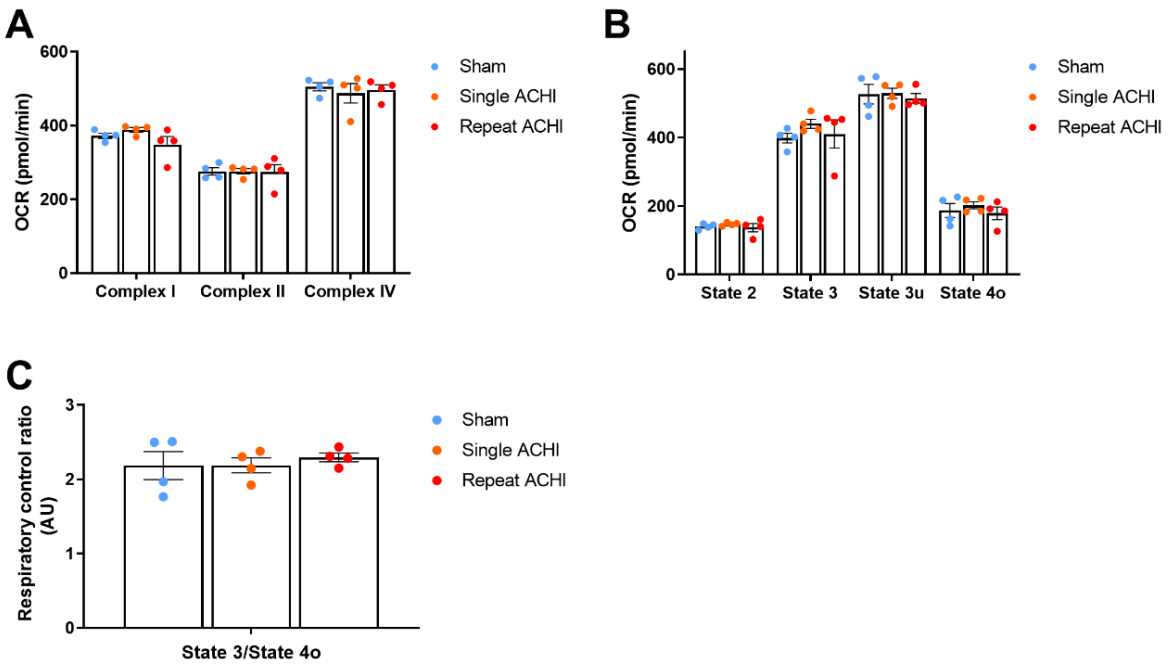

Fig. 4.5. Effect of single or repeated mTBI on isolated hippocampal mitochondria respiration at 24 h. No differences were revealed in the **A)** ETF assay, **B)** coupling assay or **C)** RCR. n=4/group. Data are presented as mean ± SEM.

4.4. Discussion

The utility of ^{1}H-MRS as a biomarker of human mTBI has been hypothesised by some researchers [24, 34, 346, 347], however few have utilised the technique in clinically relevant rodent models of mTBI. Consequently, the underlying neuropathology causing potential metabolite alterations identified in the ^{1}H-MRS is poorly understood. Increased understanding of the underpinning mechanisms may lead to the discovery of therapeutic targets and biomarkers of injury. In the current study, an increase in Gln and decrease of Glc in the hippocampus using the ^{1}H-MRS was observed 24 hours after repeat, but not single, mTBI in rats. Furthermore, the current study found that these metabolite alterations were not accompanied by mitochondrial dysfunction in the ipsilateral hippocampus after single or repeated mTBI.

4.4.1 ACHI produced acute signs of single and repeated injury

In **Chapters 2** and **3**, the beam task immediately after impact as an indication of injury confirmation, consistency, and a measure of sensorimotor deficits. However, whilst **Chapter 2** found sensorimotor deficits immediately after a single mTBI with resolution at 24 h, it was unknown how long these deficits were present for. As such, the current study had rats perform the beam task at 1- and 4-hours after a single or repeated mTBI. This revealed significant beam deficits in repeated mTBI rats across both timepoints, but none following single injury. This finding is consistent with some clinical evidence that those with a history of mTBI may display prolonged subtle gait and balance impairments [303, 304]. Whilst no changes were detected in sensorimotor deficits, metabolites or mitochondrial bioenergetics after a single mTBI, this does not mean an injury did not occur.

Based on the recent injury definitions provided by the international consensus of video signs of concussion in professional sports [345], the current study used a video observation protocol to confirm mTBI in rats immediately post impact. Video signs of mTBI were observed immediately following impact, confirming that injury occurred.

4.4.2 ^{1}H-MRS detected some neurometabolite changes acutely after single and repeated mTBI

There is some evidence indicating that ^{1}H-MRS may have potential to be utilised to help diagnose and monitor clinical mTBI. Previous pre-clinical studies have shown the mTBI can acutely alter some neurometabolite concentrations observed with the ^{1}H-MRS after a single [253, 347] or repeated mTBI [212]. Herein I will review my findings from ^{1}H-MRS and how these compare to the literature.

Glu and Gln: Due to the similarity of their molecular structures, resulting in similar peaks on the spectra, early clinical studies traditionally combined the absolute

concentrations of Glu and Gln [33, 332, 342]. However, this method does not allow researchers to distinguish between Glu and Gln, which may undergo opposite directions of concentration change [348]. Recent improvements in *in vivo* magnetic resonance scanners and the development of advanced spectral-editing methods now make it possible to evaluate Glu and Gln separately [348]. Whilst the ^{1}H-MRS did not find a significant decrease in Glu concentration between groups in the current study, it did observe a marked increase of Gln in the hippocampus, 24 hours after repeated, but not single, mTBI. This may be due to the astrocytic uptake of excess Glu, released acutely in response to TBI [333, 341] which is then converted and stored as Gln to help maintain the homeostasis of these metabolites [348, 349]. Future studies are however required to test this hypothesis and to further understand the implications single and repeated mTBI have on altered glutamatergic metabolism.

Glc: The current study also detected a reduction in Glc concentration in the hippocampus following repeated mTBI, and to the best of my knowledge, is the first study to report Glc concentration after mTBI using ^{1}H-MRS in rodents. This decrease may be due to reduced Glc uptake, as observed in a number of animal and human TBI studies [94, 189, 350-352]. Another explanation for this decrease may be due to hyper-glycolysis (i.e. increased breakdown of cellular stores of Glc to produce ATP) to compensate for potential ATP depletion due to repeated brain injury [94]. Whilst evidence of hyper-glycolysis has been found in more severe TBI studies [94], additional research is required to confirm the presence of hyper-glycolysis in mTBI and further investigate whether hyper-glycolysis occurs after single and/or repeated mTBI and is responsible for the decreased Glc levels observed in this model of mTBI.

NAA, Cr+PCr and GPC+PCh: Finally, the current study did not detect alterations between injury groups, in NAA, Cr+PCr, or GPC+PCh. Whilst these findings are consistent with some clinical studies [335, 353] and a rodent study [212], they were in

contrast to the emerging hypothesis that NAA is a potential biomarker of mTBI and injury severity [188, 346]. A number of clinical studies have found a reduction of NAA/Cr following single mTBI [24, 346], repeated mTBI [188] and in athletes with PPCS [46]. However, there are numerous differences within the abovementioned studies to the current study (including species, sex, time of analysis, voxel size and placement, scanning equipment and method of analysis) which may have contributed to contrasting findings. Additionally, although a hemisphere by injury interaction was not found for NAA, notably, an ANOVA of the ipsilateral hippocampus only revealed significantly decreased NAA concentration compared to shams. As such, ipsilateral changes in NAA were possibly present, however increased sample sizes may have been required with the study and analyses protocol.

Whilst the current study focused on hippocampal gray matter, (due to my proteomic findings in **Chapter 3**), many clinical and preclinical studies exclusively evaluate white matter regions (e.g., CC and frontal lobes). A previous study found decreased white matter Cr+PCr levels in mTBI patients compared to controls with no differences between groups in gray matter [36], suggesting metabolic response to mTBI may vary in different brain regions.

4.4.3 Single and repeated mTBI did not acutely change mitochondrial bioenergetics

Although mitochondria are known to have a critical role in cellular metabolism, the underlying clinical mTBI pathologies resulting in metabolite alterations seen in the ^{1}H-MRS are still poorly understood. As such, we aimed to investigate whether mitochondrial bioenergetics directly affected neurometabolic alterations. The ipsilateral hippocampus was chosen as the region of interest (ROI) due to the changes in hippocampal protein related to energy metabolism revealed in **Chapter 3**. Additionally, previous studies have found that the hippocampus is susceptible to changes after preclinical and clinical mTBI [313, 354]. However, the current study found no changes in

OCRs of ETC complexes I, II and IV, mitochondrial respiration States 2, 3, 3u and 4o at 24 hours after single and repeated mTBI. Furthermore, there were no significant findings in the RCR, indicating that oxidative phosphorylation was not altered between groups at 24 hours post-final ACHI [355]. These findings are somewhat in contrast to those of Hubbard et al., who found a significant decrease in hippocampal mitochondrial State 3 respiration, 48 hours after closed-head mTBI in mice, with a secondary injury delivered at 48 hours prolonging cortical mitochondrial impairment [190]. However, differences in time-points, injury inductions, species, anaesthesia, surgery, velocity of impactor, and head restraint, may have contributed to the differences in mitochondrial findings.

Notably, we found that rats subjected to ~ 50 - 60 min of ^{1}H-MRS prior to tissue collection, had highly variable functionality in the ETC complexes and mitochondrial states of respiration, whilst rats that did not undergo ^{1}H-MRS had more consistent results. This is likely due to unavoidable variables associated with *in vivo* ^{1}H-MRS including combining multiple cohorts of rats for analysis due to the limited number of rats able to be scanned per day and varied resting times after scans before tissue collection. Additionally, prolonged exposure to isoflurane for scans before tissue collection may have contributed to variable results. A previous study found that exposure to 2% isoflurane for 3 hours altered the mitochondrial permeability transition pore and decreased the mitochondrial membrane potential and ATP levels in mouse hippocampal cells [356]. These findings suggest that the prolonged isoflurane exposure in the current study may have compromised mitochondrial function and the ETC. This was reflected in the current study, in which I found reduced OCR levels of cohorts exposed to prolonged isoflurane in the ^{1}H-MRS compared to those that were not. Moreover, as this study only used one anaesthetic compound, it is unknown whether increased cerebral lactate acidosis in response to isoflurane exposure during *in vivo* ^{1}H-MRS could have also confounded cerebral metabolite levels and tissue analysis.

4.5.4 Limitations

The current study did not find mitochondrial dysfunction to be associated with the alterations seen in the ^{1}H-MRS using this rat model of mTBI at 24 h. However, there were some limitations that should be considered, and future investigations should further explore using this clinically relevant model of mTBI. Firstly, the COVID-19 pandemic restrictions on university research did not allow for further testing and, as such, the sample size for the current study was lower than originally planned, and may have been underpowered. For example, we observed potential trends in some of the metabolites (e.g. NAA), but considering the substantial variability within each group, it is possible that increased sample sizes may have produced different findings. Although this study focused on investigating metabolic changes at 24 hours post-injury, the peak of metabolite alterations may have been missed, with one rodent study observing metabolite changes 3 days post-single closed-head mTBI [253], and another finding altered State 3 mitochondrial respiration 24- and 48-hours after a single mild closed-head injury [190]. Another potential confounder is the relative tissue contributions within the voxel during ^{1}H-MRS scans. Due to the small region size of the rat hippocampus, the voxel may have included some CC and CSF, and as such, it is possible that metabolite changes may have included surrounding regions, potentially increasing variability and reducing accuracy of metabolite measures. Lastly, the current study only utilised male rats, however, evidence suggests that there are sex-based differences in h-tau [357], inflammatory pathology [358] and neurometabolite concentrations [253] in response to TBI. As such, further investigation into possible sex-based changes in metabolic pathology in response to single and repeated mTBI is warranted.

4.5.5 Conclusions

Although the current study detected altered Gln and Glc post-repeated mTBI in the hippocampus using ^{1}H-MRS, this was not associated with hippocampal mitochondrial

dysfunction at 24 hours after injury. Limited literature on ^{1}H-MRS prevents this neuroimaging technique from being a feasibly applied biomarker of mTBI and as such, more research using clinically relevant rodent models of mTBI are needed to understand the underpinning neuropathological changes responsible for these alterations.

Chapter 5: Conclusions and Future Directions

5.1 Summary of findings

This thesis aimed to develop and characterise a novel rat model of mTBI and use it to provide novel insights into the neurobiological and neurobehavioural effects of single and repeated mTBI. The first study (i.e. **Chapter 2)** of this thesis aimed to characterise the effects of a single injury using a modified version of the novel surgery and anaesthetic-free ACHI model in rats. These experiments revealed a rapid onset of sensorimotor deficits within 1-2 minutes post-ACHI and reduced exploratory behaviour at 20 minutes that resolved within 24 hours after injury, along with spatial memory deficits that were present at 5 minutes and 24-hours before resolving at 48-hours post-injury. Moreover, I found evidence of region-specific changes in microglia and astrocyte reactivity that appeared to peak at 3 days post-injury. Notably, these changes were present when rats no longer had behavioural deficits, potentially indicating that glial reactivity may persist beyond symptoms and represent a form of ICV to repeated mTBI. In addition, to the best of my knowledge, this study is the first to provide a sequence of detailed traditional neurobehavioural assessments within 20 minutes of TBI in rodents.

Chapter 3 aimed to use the novel mTBI rat model characterised in **Chapter 2** to further the understanding of the acute and chronic effects of repeated mTBI. These experiments found evidence of prolonged sensorimotor impairments in rats given four ACHIs, along with subtle changes in spatial memory in the acute stages only. In addition, this study found no evidence of changes in locomotion or anxiety-like behaviour at 24 hours and 3.5 months. Considered together, these findings indicate that although behavioural changes after repeated ACHIs did appear to persist for longer than a single ACHI in **Chapter 2** overall, the behavioural changes were relatively mild when compared to several rodent-based repeated mTBI studies in the literature. Nonetheless, this chapter found evidence of an array of biological and pathological changes present in both the acute and chronic stages of injury. Firstly, I found a profound elevation in serum levels of

the axonal damage marker NfL at 7 days post-injury. Notably, serum NfL levels correlated with sensorimotor deficit metrics, providing novel evidence that serum NfL may be a useful biomarker of injury severity in rodent models of TBI. However, I found no changes in serum NfL levels in repeated mTBI rats compared to sham rats at 3.5 months, indicating that axonal injury / degeneration was not likely to be ongoing at this chronic stage of injury. Nonetheless, advanced DTI analysis revealed that at this time-point there were indeed widespread reductions in white matter integrity, likely indicating that axonal damage induced by repeated mTBI persisted to chronic stages of injury. Finally, this chapter aimed to increase understanding of the neurobiological changes induced by repeated mTBI through use of unbiased mass spectrometry of the hippocampal proteome. I found evidence of several proteomic changes at acute and chronic stages of injury, including proteins suggestive of disrupted Ca^{2+} homeostasis, energy metabolism, cerebrovascular damage, altered glial reactivity, axonal and neuronal function. Overall, this chapter provided novel pathological findings which may help inform research on new treatment targets and biomarkers for mTBI.

Finally, given that the majority of significantly altered hippocampal proteins were related to mitochondrial metabolism acutely after repeated mTBI in **Chapter 3**, **Chapter 4** aimed to further investigate the neurometabolic changes after single and repeated mTBI, using ^{1}H-MRS, and whether changes were driven by mitochondrial dysfunction. Sensorimotor deficits were observed at 1-and 4-hours after-final injury in repeatedly injured rats compared to sham and single mTBI, providing evidence that repeated ACHIs did result in cumulative damage. ^{1}H-MRS revealed increased Gln and decreased Glc in the hippocampus of repeated mTBI rats compared to sham at 24 hours post-final injury. However, contrary to my hypothesis, function of mitochondria isolated from the ipsilateral hippocampus did not appear to be affected by single and repeated mTBI,

providing evidence that metabolic disruption in these two metabolites may not be driven by mitochondria at this time-point.

This thesis characterises a novel clinically relevant rat model of mTBI and adds valuable neurobehavioural and neuropathological insights into single and repeated mTBI. Nonetheless, further investigations are warranted to understand other pathophysiological mechanisms of injury using the ACHI model. In particular, I suggest that this clinically relevant rat model will be ideally suited to increasing understanding of mTBI neurobiology, in order to assist in the identification of translatable biomarkers and treatments strategies for clinical mTBI.

5.2 Future Directions

5.2.1 Neuroinflammation

Inflammation is thought to be an influential component and pathological hallmark of TBI, and **Chapter 2** of my thesis provided further evidence that even mild forms of TBI can result in increased astrocyte and microglia reactivity. However, more experiments are needed to understand the significance of the transient nature of glial disturbances after a single mTBI, including how this may contribute to the potential period of ICV to exacerbated and/or persistent effects of repeated mTBI. Herein, I will outline some potential areas for follow-up studies investigating neuroinflammation in this context.

A complex of proteins, called the nucleotide-binding oligomerisation domain-like receptor pyrin domain-containing-3 (NLRP3) inflammasome, [359] has been consistently found to be expressed in microglia and astrocytes [360], in which my group has recently postulated to have a role in neuroinflammation after mTBI [361]. The upregulation of NLRP3 has been observed in the neuroinflammatory pathology of preclinical moderate

TBI [360, 362] and severe clinical TBI [363, 364]. Although the NLRP3 inflammasome has not been directly investigated in mTBI pathology, it is hypothesised that mTBI may prime and/or activate the inflammasome and have serious implications in repeated mTBI [361]. As such, I hypothesise that although a single ACHI resulted in only transient glial changes, prolonged glial disturbances may occur if repeated injuries are experienced while the inflammasome remains in a primed state. Although I did not directly assess glial reactivity in **Chapter 3**, I did observe some proteomic changes indicating that hippocampal glial disturbances were indeed persistent to at least 7 days post-injury.

Whilst **Chapter 2** utilised immunochemistry to reveal region specific neuroinflammation in the brain after single mTBI, such analysis was not performed after repeated mTBI for **Chapter 3**. As discussed in **Chapter 1**, other rodent studies have shown that repeated mTBI can exacerbate and prolong neuroinflammation, [132, 172, 179, 180], however, many of these studies used suboptimal models that involved confounding variables such as anaesthesia, surgery and/or craniotomy. As such, future studies should incorporate immunochemistry after repeated mTBI at acute and chronic timepoints using this clinically relevant model of injury. Moreover, it should be acknowledged that detailed glial morphological analysis, or additional techniques such as flow cytometry, are likely to provide further insights into the nature of glial changes after single and repeated ACHIs. In addition, future studies could pinpoint the exact time of peak neuroinflammatory activation and resolution by using a translatable measure such as serial TSPO-targeted PET imaging to monitor the neuroinflammatory response after a single ACHI. This imaging technique has been utilised in preliminary clinical studies in which have found that those with an extensive history of mTBI had an increased expression of TSPO [178, 365]. This advanced imaging technique has huge potential to help further understand the temporal *in vivo* neuroinflammatory profile of a mTBI in controlled preclinical studies. Given that anaesthetics has been shown to inhibit activation

of microglia [125], the ACHI model may be the most ideal method to induce injury when investigating mTBI inflammation pathology. Together with TSPO-targeted PET imaging, a follow up study using the ACHI could provide new clinically relevant understanding of the period of ICV and inform inflammatory resolution.

Furthermore, there are a number of neuroinflammation-related serum biomarkers postulated to have some utility for post-injury evaluation and predicting cognitive outcome. A clinical study found that serum cytokines Il-1β, IL-6 and C-C Motif Chemokine Ligand 2 (CCL2), were acutely elevated in mTBI patients compared to controls, with elevated levels correlating to post-concussive symptoms [366]. Additionally, CCL2 levels remained elevated for at least 3 months post-injury [366]. Accordingly, future studies could utilise the ACHI model and combine neurobehavioural tasks and evaluation of serum and brain tissue to investigate whether elevated serum cytokines are derived from the brain, and if they are useful serum biomarkers to evaluate and predict symptom outcome after mTBI.

5.2.2 Effects of Stress on repeated ACHI

Arguably, athletes and military personnel are often under physical and/or psychological stress when sustaining mTBIs. Environmental stress has been hypothesised to be a determinant for worse outcomes in military personnel surrounding brain injury [367]. Although rats were acclimated to the restraint protocol before undergoing injuries, it is possible that these rats had elevated stress levels during subsequent awake injuries. Even though a recent study found stress induced by forced swim testing 30 min prior to a single mTBI improved cognitive outcomes in mice [368], the majority of rodent studies have found physical stressors such as inescapable electric foot shocks [369, 370], tail shocks [371] and immobilisation [372] to cause increased inflammatory markers in the brain .

The advantage of the ACHI model is the elimination of anaesthesia, as it has been shown to alter several different pathologies [124, 126-128]. Whilst the restraint to induce an ACHI occurs for significantly less time (under 1 min) than the aforementioned immobilisation study (2 h in a Plexiglas tube), the implications that stress has on injury should be considered. As such, future studies could introduce an anaesthetised sham and mTBI group along with awake groups to further compare the behavioural and pathological confounds of anaesthesia and stress using the ACHI model.

5.2.3 Markers of neurodegenerative disease?

Due to time and financial constraints, preclinical studies rarely extend to timepoints longer than 12 months post-injury [252], and consequently, given the inherent difficulties in studying the long-term effects of mTBIs in humans, the current understanding of chronic pathological mechanisms of repeated mTBI remains poor. Although **Chapter 3** found evidence of pathological changes at 3.5 months after repeated mTBI exposure in rats, resolution of serum NfL levels indicated that axonal damage was not ongoing. Whilst rat months may reflect years in the human lifespan [88], future studies could extend to more chronic timepoints in rodents (e.g. 12-18 months). Such long-term studies may be more equivalent to late life in humans, and the typical age of disease onset, to study the link between neurodegenerative disorders and repeated mTBI exposure. In addition, there are a number of key aspects of neuropathology linked to repeated mTBIs that warrant further investigation using the clinically relevant rat model of this thesis.

H-tau: As briefly mentioned in **Chapter 1**, the microtubule-associated protein tau, is particularly abundant in neurons and vital for cell function by promoting the assembly of microtubules and stabilising their structure [167]. However, repetitive head injuries are thought to trigger the accumulation of h-tau, causing the protein to form astrocytic tangles and/or neurofibrillary tangles (NFT) instead of binding to microtubules

and therefore inhibiting and disrupting microtubule assembly [373]. The presence of h-tau deposits, due to repeated mTBIs, in neurons and astrocytes are a unique pathological hallmark of CTE and AD [8, 141, 162, 164]. Multiple post-mortem studies report abnormal aggregation of tau in the cerebral cortex, subcortical nuclei and brainstem, as such AT's and NFT's are considered primary indicators of CTE [77, 373]. Furthermore, these clusters have been found within the perivascular spaces of veterans exposed to repetitive blast TBI, football players and professional wrestlers with repetitive head trauma [374] supporting the hypothesis that h-tau aggregation may be correlated with repeated mTBI and eventually lead to the development of CTE. However, clinical neuropathological evidence of h-tau has only been gathered from post-mortem examinations of those with a history of mTBI. As death often occurs in late life, it is unknown how soon h-tau deposits accumulate, whether it can develop after sub-concussive impacts, and exactly what pathology leads to the development of h-tau. As such, controlled pre-clinical investigations are needed to reveal these unknowns.

A number of animal studies have shown that repeated mTBI can lead to chronic h-tau [135, 136, 141, 174, 307]. For instance, one study found that a single mTBI induced transient h-tau, which was exacerbated and prolonged following 2 and 3 repeated injuries [174]. However, the increased phosphorylation of tau in response to repeated mTBI has not been successfully replicated in all studies [132, 173, 375]. Xu and colleagues found no evidence of h-tau in mouse brains at 10 weeks despite high frequency (3 mTBIs/day with a total of 12 mTBIs) repetitive mTBI [173], whilst Mouzon et al. and Mannix et al also found no increases in h-tau at 6 months after repetitive closed-head mTBI [132, 375]. Moreover, the injury model and severity of injuries in some studies may exceed a mild injury.

Overall, the link between repeated mTBI and the development of AD and CTE to h-tau is still relatively new, and success in replicating the accumulation of h-tau in pre-

clinical models has been variable. As previous studies have found anaesthetics to alter tau phosphorylation [127, 128], the anaesthetic- and surgery-free ACHI may be the ideal rodent mTBI model to investigate the potential presence of h-tau in multiple regions of the brain by utilising western blotting for phosphorylated tau at this timepoint in future studies.

TAR DNA-Binding protein 43: TAR DNA-Binding protein 43 (TDP-43) is a transcriptional repressor in the CNS which regulates the binding to deoxyribonucleic acid (DNA) or ribonucleic acid [376] and splicing of pre-mRNAs [377]. The hyperphosphorylation of TDP-43 can result in a toxic gain and/or loss of TDP-43 function, and ultimately lead to neurodegeneration. TDP-43 has previously been established as a pathological marker of the deadly progressive neurodegenerative disease, ALS, affecting motor neurons in the spinal cord and brain [378, 379]. Previous studies have suggested that development of ALS may be from genetic or environmental triggers, including exposure to brain or spinal injury. Studies have shown that ALS mortality is reportedly higher in athletes, including soccer players [380, 381] and American football players [10]. However, whether TBI can cause the accumulation TDP-43 and therefore ALS progression remains controversial [212].

Despite links found between TDP-43 and CTE patients, few preclinical studies have investigated TDP-43 pathology after repeated mTBI. A study by McKee and colleagues found aggregates of TDP-43 proteinopathy in 85% of 68 CTE brains post mortem [164]. However, without a reliable method to screen for such pathologies in living individuals, investigators remain confined to observing post-mortem neuropathology, greatly limiting the understanding of the mechanisms of this disease. Future preclinical studies of repeated mTBI in this model would allow researchers to add to the limited literature regarding TDP-43 and CTE.

Overall, future longitudinal pre-clinical research is required to assess the chronic development of h-tau and TDP-43 after repeated mTBI, and to help further understand the aetiology of these neurodegenerative diseases.

5.2.4 Lipid metabolism?

Using unbiased mass spectroscopy, **Chapter 3** revealed several proteomic alterations which may provide novel insight to help further understand the neuropathology of repeated mTBI. Briefly, acute alterations consisted of proteins associated with Ca^{2+} homeostasis, energy metabolism, cerebrovascular damage, gliosis, and endothelial cell integrity, whilst chronic proteomic findings included proteins involved in axonal and neuronal development and function.

As discussed in detail in **Chapter 3**, the majority of altered proteins were associated with β-oxidation of fatty acids at the acute timepoint after repeated mTBI, with further investigations required to understand these mechanisms. Recently, disturbances to cellular lipid metabolism have been postulated to be a pathological hallmark of AD for therapeutic intervention due to their involvement in blood brain barrier function, APP processing, inflammation, oxidation, myelination, energy homeostasis and more [382]. mTBI also disrupts many of the same pathological mechanisms in the brain. One study investigated plasma lipidomic profiles of military personal with mTBI and/or post-traumatic stress disorder in those carrying the APOE (a mediator of lipid and cholesterol transport) with the ε4 allele. Whilst the study found varied results in the PTSD-only group, phospholipid classes in the plasma of mTBI and mTBI+PTSD were significantly altered, and that the APOE ε4 has influence over lipid metabolism [383, 384]. Future studies could utilise untargeted lipidomic analysis of brain tissue and plasma to uncover lipid species affected by mTBI. Such studies could uncover potential biomarkers and therapeutic targets for mTBI.

5.2.5 Glu excitotoxicity?

As briefly mentioned in **Chapter 1**, Glu is the primary excitatory neurotransmitter of the CNS [33, 36]. The release of glutamate occurs within minutes of brain injury, and thought to be a crucial trigger of the metabolic cascade [385]. Evidence of the indiscriminate release of Glu leading to excitotoxicity and potentially cell death has been found acutely in severe TBI pathology [386-388], however, clinical trials on glutamate antagonists to combat excitotoxicity have failed to date [389, 390]. Glu excitotoxicity likely occurs after mTBI [28]. Using animal models of mTBI, studies have found Glu increases at 10 minutes [92, 391], with one finding resolution after 20 minutes [391]. However, the variations and severities of the preclinical models used may be questionable, with few studies using clinically relevant animal models of repeated mTBI to study this pathology [93]. As such, the rodent ACHI may be an ideal model to further investigate the time of emergence and resolution of Glu excitotoxicity after single mTBI, and its potential exacerbation after repeated injury.

As seen in **Chapter 4**, the ^{1}H-MRS did not detect significant changes Glu concentrations after repeated mTBI compared to sham rats. One explanation for unchanged Glu is that ^{1}H-MRS scans cannot distinguish between intra- and extracellular Glu levels within the voxel. As such, Glu concentration may have been detected in both the presynaptic vesicles and the synaptic space, likely explaining the lack of differences observed between sham, single and repeated mTBI rats using this technique. Microdialysis is an *in vivo*, minimally invasive technique used to quantify neurotransmitters (e.g. Glu and GABA) without loss of life [93]. Future studies could utilise microdialysis to confirm whether the ACHI model acutely induces the indiscriminant release of extracellular glutamate, as described in the mTBI literature outlined above [92, 93, 391].

5.2.6 Sex-based pathological differences

As I only utilised male rats, the findings in this thesis cannot be generalised to females. Notably, growing evidence suggests that there are many sex differences in the neurobehavioural and neuropathological responses to mTBI.

For example, a previous sex-based study in our group observed that a single mTBI altered social dynamics more severely in females than males [392]. Our group also found that only male rats had short-term working memory impairments whilst only females displayed depressive anxiety-like behaviour after repeated mTBI in a separate study [226]. Evidence of sexual dimorphism in neuroinflammation has also been demonstrated by our group and others. Our group found that females display increased GFAP markers after repeated mTBI in rats [226], whilst another study found sex-based differences in the functional activation of microglia after a moderate-severe TBI in mice [358]. Additionally, another preclinical study revealed that aged female mice have lower histological phosphorylated tau than males after repeated mTBI [357], whilst our group has found increased tau expression in the CC of female compared to male rats after repeated mTBI [226]. Lastly, sex-based metabolic differences have also been found after mTBI. A pre-clinical study by Lyons et al. found a reduction in NAA, PCr, GPC+PCh and gamma aminobutyric acid (GABA), at three days after a single closed-head mTBI in female, but not male mice [253]. Furthermore, the changes in NAA, PCr, and GABA remained decreased at 28 days after injury [253]. Although there are numerous differences between this thesis and other studies (including mTBI model, injury region, species, time post-injury, scanning equipment and method) more studies are needed to understand the sex-based pathophysiological differences in response to single and repeated mTBI.

5.2.7 Consistency vs. Heterogeneity

Translation of pathobiological insights, biomarkers and therapies from pre-clinical mTBI models to clinic mTBI is widely acknowledged to be challenging, with the significant clinical heterogeneity commonly seen as a barrier to successful research translation.

As mentioned throughout this thesis, animal models allow researchers to minimise variables seen in clinical settings, and therefore, isolate behavioural and pathobiological changes produced by utilising different types and severities of mTBI. By controlling for multiple variables, animal studies can attempt to replicate the effects of specific environmental factors, genetic predispositions, age and sex-specific differences on mTBI behavioural and pathological outcomes [11], making studies and injuries more reproducible. However, replicating a heterogenous population in animals may aid in ensuring that potential biomarkers and treatments can be translated to a clinical population that typically features a greater degree of variation in injury mechanisms and severities. As such, closed-head models that feature a greater degree of variability, such as the ACHI used in this thesis, may actually provide more clinically relevant and applicable insights than models traditionally known to have greater degree of reproducibility.

5.2.8 Other brain regions

Due to the region-specific inflammatory findings in **Chapter 2**, surrounding the ipsilateral CC and its adjacent regions, notably the hippocampus, this thesis intensively investigated the effects of a single or repeated mTBI on the ipsilateral hippocampus using the ACHI. However, **Chapter 4** revealed that repeated injury induced some acute metabolic hippocampal changes detected in the ^{1}H-MRS in both hemispheres of the brain. Additionally, TBSS in **Chapter 3** at the chronic timepoint revealed that repeated ACHI produced a widespread diffusive injury in rats. As such future studies should further

investigate the effects of single and repeated ACHI in multiple ipsilateral and contralateral regions of the brain.

5.3 Conclusion

In conclusion, this thesis adds important neurobehavioural and neuropathological insights into single and repeated mTBI using a clinically relevant model, the ACHI. The findings and model of mTBI have significant value in revealing more translatable pathological treatment targets and biomarkers to improve clinical management of injury. Moving forward, it will be important to recognise the plethora of pathophysiological aspects to single and repeated mTBI that have not been investigated using clinically relevant animal models. As such, future investigations are clearly needed and will likely unveil more potential biomarkers and treatment targets for mTBI.

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Appendix A: Modification of the ACHI model

Modification of the ACHI model

The ACHI model was originally developed by Meconi et al. at The University of Victoria (Canada), with this version of the model found to induce acute neurological and white matter changes following repeated injury in juvenile rats [134, 255]. In order to induce single and repeated injuries on larger adolescent rats in my thesis, I modified a number of aspects of the original ACHI model using cadaver rats before finalising changes and commencing experiments for **Chapter 2, 3** and **4**.

The changes made to the ACHI model and the rationale for these changes are as follows:

1) The design for the original custom 3D printed acrylonitrile butadiene styrene plastic helmet (Figure. A.1.A) used on juvenile rats in Meconi et al. was provided by the authors of and printed using stereolithography plastic in varying larger sizes to accommodate for the continued growth of adolescent rats.
2) Meconi et al. featured a rubber surface on the impactor tip. In order to increase kinetic energy transfer to the helmet, and thus transfer increased force to the skull of the larger rat, we opted to instead utilise a cylindrical stainless-steel tip as shown in **Chapter 2**, Figure. 2.1.
3) To ensure the flat impactor tip would make contact perpendicularly (i.e. flush) on the helmet impact site, the impactor was set at 14° from the vertical position, rather than completely at the vertical position as in Meconi et al. [134].
4) Initial studies in cadaver rats revealed that using this configuration, the larger plastic helmets occasionally fractured upon impact. As such, I experimented further with a modified design of SLA helmet featuring a thicker horizontal band (Figure. A.1.B). This helmet did not appear to fracture upon impact; however, pilot testing with force sensor film revealed that substantial reduction in force exerted on the scalp/skull when compared to direct impacts without a helmet. Moreover, there was concern that

repeated impacts may result in a gradual weakening of the plastic material. As such, after consultation with biomechanical experts we decided to alter the helmet material to 3D printed stainless steel (Figure. A.1.C). By using a steel impactor tip and steel helmet, we hypothesised that the kinetic energy, delivered by the steel impactor, was able to be maintained when travelling through the steel helmet to deliver a diffuse injury directly to the parietal bone without skull fracture. Pilot testing using force sensors confirmed substantially more helmet to scalp/skull force transfer using this model, and no skull fractures were detected.

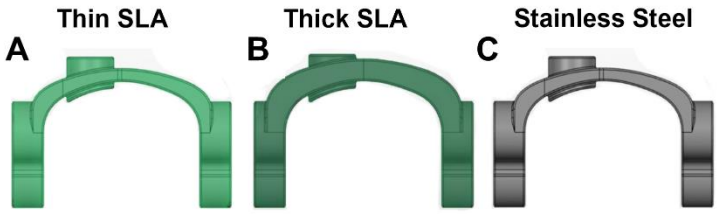

Figure. A.1. Helmet modifications for the ACHI model. Various modifications were made before commencing experimental chapters. **A)** the original dimensions of the 3D ACHI helmet were printed in larger sizes for larger rats. Due to fracturing of the band, **B)** thick SLA helmets were printed instead. However, upon further fracturing, the current study used **C)** stainless steel 3D printed helmets.

5) Although the number of experimenters required for injury delivery in the original ACHI is not specified in Meconi et al. [134], my supervisor (Dr Stuart McDonald) was trained in the model at The Univerity of Victoria, and confirmed that only one experimenter was used. In attempt to increase the efficiency and rigour of the model in our hands, I implemented a procedure involving two researchers. One experimenter was needed to carefully restrain the rat using the DecapiCone (Braintree Scientific Inc., MA, USA), and close off the opening behind their haunches with their hand and hold the rat in place on the foam platform. The second experimenter placed,

positioned, and secured the helmet onto the rat, checked that impactor tip and site were perpendicular before setting off the impactor for injuries.

Appendix B: Author Contributions

Author Contributions

In the case of **Chapter 2**, the nature and extent of my contribution to the work was the following:

Contributor	***% of Contribution***	***Statement of Contribution***
Louise Pham	57%	Codeveloped injury model; designed experiment; conducted injuries and behavioural tests; analysed behavioural outcomes; collected tissue; cut sections, completed immunohistochemistry staining and analysis; drafted the manuscript
Sandy R. Shultz	2%	Conceptualised and designed injury model; critically reviewed and edited the manuscript
Hyun A. Kim	3%	Conducted immunofluorescence staining; provided knowledge for sectioning; critically reviewed and edited the manuscript
Rhys D. Brady	2%	Collected tissue; critically reviewed and edited the manuscript
Ryan C. Wortman	2%	Conceptualised and designed injury model; critically reviewed and edited the manuscript
Shannyn G. Genders	3%	Conducted behavioral testing; critically reviewed and edited the manuscript
Matthew W. Hale	2%	Provided knowledge for tissue collection and sectioning, critically reviewed and edited the manuscript
Ross D. O'Shea	2%	Provided knowledge of immunofluorescence staining and analysis; critically reviewed and edited the manuscript
Elvan Djouma	2%	Provided knowledge for behavioural testing and analysis; critically reviewed and edited the manuscript
Maarten van den Buuse	2%	Provided knowledge for behavioural testing and analysis; critically reviewed and edited the manuscript
Jarrod E. Church	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Brian R. Christie	2%	Conceptualised and designed injury model;

		critically reviewed and edited the manuscript
Grant R. Drummond	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Christopher G. Sobey	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Stuart J. McDonald	15%	Codeveloped injury model; conceptualised, designed project; assisted with injuries and behavioural tests; critically reviewed and edited the manuscript

In the case of **Chapter 3**, the nature and extent of my contribution to the work was the following:

Contributor	***% of Contribution***	***Statement of Contribution***
Louise Pham	60%	Designed behavioural experiments; conducted injuries and behavioural tests; analysed all behavioural outcomes; collected tissue; interpreted proteomic data; drafted the manuscript
David K. Wright	3%	Generated and analysed MRI data; drafted MRI methods of the manuscript; critically reviewed and edited the manuscript
William T. O'Brien	2%	Generated serum data; critically reviewed and edited the manuscript
Jesse Bain	2%	Analysed raw proteomic data; critically reviewed and edited the manuscript
Cheng Huang	3%	Generated raw proteomic data; drafted mass spectroscopy methods of the manuscript, reviewed manuscript
Mujun Sun	2%	Conducted brain induction for MRI analysis; provided knowledge for MRI analysis; critically reviewed and edited the manuscript
Pablo M. Casillas-Espinosa	2%	Video analysis of injuries; critically reviewed and edited the manuscript
Anup D. Shah	2%	Generated raw proteomic data; critically reviewed and edited the manuscript
Ralf B. Schittenhelm	2%	Generated raw proteomic data; critically reviewed and edited the manuscript
Christopher G. Sobey	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Rhys D. Brady	2%	Collected brain tissue; critically reviewed and edited the manuscript
Terrance J. O'Brien	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Richelle Mychasiuk	2%	Interpreted proteomic data; critically reviewed and edited the manuscript
Sandy R. Shultz	2%	Conceptualised and designed project; critically reviewed and edited the manuscript

Stuart J. McDonald	12%	Conceptualised, designed and supervised project; conducted injuries and behavioural tests; collected brain tissue; critically reviewed and edited the manuscript

In the case of **Chapter 4**, the nature and extent of my contribution to the work was the following:

Contributor	***% of Contribution***	***Statement of Contribution***
Louise Pham	60%	Designed experiment; conducted injuries and beam tests; monitored rats during ^{1}H-MRS scans; collected tissue; analysed all data; interpreted ^{1}H-MRS and Seahorse XF Analyser data; drafted the manuscript
William T. O'Brien	15%	Conducted injuries; collected tissue; critically reviewed and edited the manuscript
David K. Wright	5%	Generated ^{1}H-MRS data; critically reviewed and edited the manuscript
Jesse Bain	2%	Collected tissue; critically reviewed and edited the manuscript
Sandy R. Shultz	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Christopher G. Sobey	2%	Conceptualised and designed project; critically reviewed and edited the manuscript
Jarrod E. Church	2%	Interpreted data; critically reviewed and edited the manuscript
Brian Drew	2%	Generated and analysed Seahorse XF Analyzer data, critically reviewed and edited the manuscript
Simon T. Bond	5%	Generated and analysed Seahorse XF Analyzer data; drafted Seahorse XF Analyser methods for manuscript; critically reviewed and edited the manuscript
Stuart J. McDonald	5%	Conceptualised and designed project; collected tissue; critically reviewed and edited the manuscript

Louise Pham

7th January 2021

Appendix C: Copyright Permissions

Copyright Permissions

This thesis resulted in work that has been published in two peer-reviewed journals (data from **Chapter 2** and **3**).

Copyright permissions to include these publications in this thesis and the university's repository (subject to proper acknowledgement) are as follows:

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Appendix D: Published Chapter 2

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Mild Closed-Head Injury in Conscious Rats Causes Transient Neurobehavioral and Glial Disturbances: A Novel Experimental Model of Concussion

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Abstract

Rodent models can provide insights into the most pertinent issues surrounding concussion. Nonetheless, the relevance of some existing models to clinical concussion can be questioned, particularly with regard to the use of surgery and anesthesia and the mechanism and severity of injury. Accordingly, we have co-developed an awake closed-head injury (ACHI) model in rats. Here, we aimed to create a temporal profile of the neurobehavioral and neuropathological effects of a single ACHI. Adolescent male rats were placed in a restraint bag and a steel helmet was positioned over the head such that the impact target was centered over the left parietal cortex. Once positioned on a foam platform, a cortical impactor was used to strike the helmet. Sham animals underwent the same procedure without impact. When compared with sham rats, those given a single ACHI displayed evidence of sensorimotor deficits and reduced exploratory behavior within the first 20 min post-injury; however, these effects were resolved after 24 h. A single ACHI impaired spatial memory on the Y-maze task at both 5 min and 24 h post-ACHI; however, no deficits were apparent at 48 h. Immunostaining revealed region-specific increases in ionized calcium-binding adaptor molecule 1 and glial fibrillary acidic protein expression at 3 days post-impact, with no differences found at either 1 or 14 days. Taken together, our findings indicate that a single ACHI results in transient neurobehavioral and glial disturbances and as such, this model may be a valuable tool for pre-clinical concussion research.

Keywords: anesthesia; astrocytes; behavior; microglia; mild traumatic brain injury; neuroinflammation

Introduction

Brain concussion, a common subset of mild traumatic brain injury (mTBI), is a serious medical and societal issue.[1] In the United States alone, the incidence of concussion is reported to be 150,000 cases per year,[2] with evidence of gross underreporting indicating that the true prevalence is much higher.[3] Concussion is particularly common in sports with the highest participation rates for adolescents and young adults,[4] and is increasingly evident in military personnel following war zone blast exposure.[5]

Although the neurobehavioral (e.g., cognitive and emotional impairments) and physical (e.g., headache, sensorimotor deficits) effects of concussion typically resolve within 7-10 days, 10-15% of individuals develop post-concussive symptoms that persist for several weeks or months.[1,6,7] Despite recent advances in neuroimaging and fluid biomarkers of concussion,[8-10] our understanding of the specific neurobiological mechanisms underlying the clinical features of concussion and post-concussion syndrome is lacking. In addition, although there is some evidence that exposure to multiple concussive impacts may lead to lingering or even chronic impairments, including neurodegenerative diseases such as chronic traumatic encephalopathy (CTE), the potential cumulative and chronic effects of repeated concussions are intensely debated and poorly understood.[11] In particular, the existence of CTE as a distinct neuropathology induced by repeated head traumas remains controversial, and factors such as the number, timing, and severity of injuries that may lead to the development of CTE are unknown.[11-13]

Given the inherent difficulties in studying the mechanisms and effects of concussion in humans, it is not surprising that much of our understanding is derived from pre-clinical models. Traditional

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TBI models, including fluid percussion injury, controlled cortical impact (CCI), and weight-drop injury have provided insights into the potential neurobehavioral and neurobiological consequences of single and repeated mTBIs.[8,14–17] Nonetheless, potential limitations to the relevance of the aforementioned models to clinical concussion have been increasingly acknowledged in the field, particular regarding use of surgical procedures (i.e., incisions and craniotomies),[18,19] and impacts to a restrained head.[14] Accordingly, in recent times a number of laboratories have developed surgery-free models featuring closed-head impacts to an unrestrained head.[8,14] For example, models featuring closed-head impacts delivered by a CCI device,[20,21] and the closed-head impact model of engineered rotational acceleration (CHIMERA),[22] represent important advances. Another potentially confounding factor in modeling concussive-like injury is the use of anesthetics and analgesics, with evidence indicating that these factors may alter brain pathology and animal behavior.[23–30] In addition, although several rodent studies have demonstrated cumulative effects of repeated mild head traumas,[8,15] the severity of injury may exceed that of a truly concussive impact with respect to structural damage or persistent neurobehavioral deficits from a single injury, or else may lack any clear single injury outcomes.

To further our understanding of the neurobiological basis and effects of concussions, it is desirable that pre-clinical studies utilize injury models that closely mimic a single concussive incident in humans. Therefore, we have further modified the recently developed awake closed-head injury (ACHI) model in rats,[31] and aimed to characterize neurobehavioral consequences of a single ACHI. In addition to eliminating the potential confounds of anesthetics and analgesics, the priority for this high-throughput model was to ensure that it resulted in the rapid onset of a range of short-lived neurobehavioral symptoms typically seen in clinical concussion. Importantly, the omission of surgery and anesthetic allowed for acute neurobehavioral testing (i.e., from 1 min post-ACHI), akin to sideline assessments in sports-related concussion.[32] Although some recent studies have incorporated acute neurological severity scale assessments shortly after impact,[31,33] to our knowledge this is the first study to conduct a sequence of detailed, traditional neurobehavioral tasks on rodents within the first 20 min of injury. Finally, given the potential role of a neuroinflammatory response in concussion symptomology,[34] we aimed to create a temporal profile of glial reactivity following a single ACHI.

Methods

Animals

Male Long-Evans rats ($n = 116$) were obtained at post-natal day (PND) 28 from Animal Resource Centre (WA, Australia). Upon arrival, rats were housed in groups of two at the La Trobe University Central Animal House, with a 12 h:12 h light/dark cycle and food and water available *ad libitum*. All procedures were approved by the Animal Ethics Committee at La Trobe University (AEC 17-06), and were within the guidelines of the Australian code of practice for the care and use of animals for scientific purposes by the Australian National Health and Medical Research Council. Upon arrival, all animals were given 48 h to acclimatize to their enclosures before experimenter contact. After this period, animals were handled 4 days per week until sham or injury procedures on PND 46.

It should be noted that some animal welfare compliance committees may be reticent to approve the study of un-anesthetized animals in TBI research. The procedure used in this study was developed and optimized under the close guidance of local animal ethics committees, including veterinarians, to carefully develop the procedures to the satisfaction of Canadian and Australian animal ethics committees. These committees have praised the fact that the model is less invasive than other mTBI models, as the removal of any incision and/or craniotomy eliminates the pain, dehydration, and risk of infection that accompany such procedures. They have also acknowledged the considerable evidence that anesthetics can compromise major outcomes of such research.

Restraint acclimatization

A disposable DecapiCone (Braintree Scientific Inc., MA) was used to restrain each rat during sham/ACHI procedures (Fig. 1A).[31] These clear plastic cones feature an opening for the snout to provide ventilation. Animals were led into the restraint cone headfirst and briefly immobilized by holding the entry point of the cone closed behind the haunches. Rats were acclimatized to the restraint cone for 3 consecutive days prior to sham/injury procedures. Twenty-four hours prior and at the time of sham/injury, as previously described,[31] rats were given a score of 1-4 based on their willingness to enter the restraint cone, as well as movement and vocalization during restraint. Following restraint acclimatization, animals were randomly assigned into sham or ACHI groups. There were no differences in restraint scores between groups.

Awake closed-head injury

Our model of concussive-like injury (Fig. 1A) was modified from a recently developed ACHI model used with PND 25-28 rats.[31,35] In our model variant, unanesthetized PND 46 rats were immobilized using a restraint cone and carefully fitted with a three-dimensional printed steel helmet (Fig. 1B), such that the target impact site was positioned over the left parietal bone. The interior surface of the helmet target was engineered to fit the curvature of the scalp/skull. An elastic band was strapped under the head to secure the helmet in place during injury. The gathering of the bag was performed only when the rats had voluntarily reached the complete end of the narrow bag, which ensured that the scalp was pulled taut. Throughout the study, one individual researcher was responsible for animal restraint and gathering of the plastic bag, with the second researcher responsible for helmet fitting/placement. Subjects were carefully lowered onto a foam platform (3″ thick Super-Cushioning Polyurethane Foam Sheet; McMaster-Carr, OH) so that the helmet target was directly underneath a steel impactor tip. A CCI (Leica Biosystems, IL) device was connected to a stereotaxic frame and an electromagnetic piston to drive the impactor tip (velocity of 6.5 m/sec; extension depth of 10 mm; dwell time of 100 msec) at a 14° angle onto the helmet target. Impact velocity and dwell times were similar to a recent model of closed-head concussive-like injury in rats using a CCI device[20] and the ACHI model from which our model was adapted,[31,35] and were the upper limit of the equipment. The extension depth was matched by the buffering capacity of the foam platform, and the 14° angle ensured that the impact tip was perpendicular to the helmet surface. In addition to allowing for a clear impact target, the steel helmet served to reduce risk for skull fracture by diffusing the force across the parietal bone. No skull fractures were observed in this study. Sham procedures were identical to those for the ACHI, except that the impact was triggered adjacent to the animal's head. The total duration of restraint during each procedure was less than 1 min. Animals were removed from the restraint cone immediately post-impact for neurobehavioral assessments.

Neurobehavioral testing

Rats underwent neurobehavioral testing at <20 min, 24 h, and/or 48 h, and were assigned into four groups (Table 1) to avoid the potential confound of repeat testing in the open field (OF), elevated

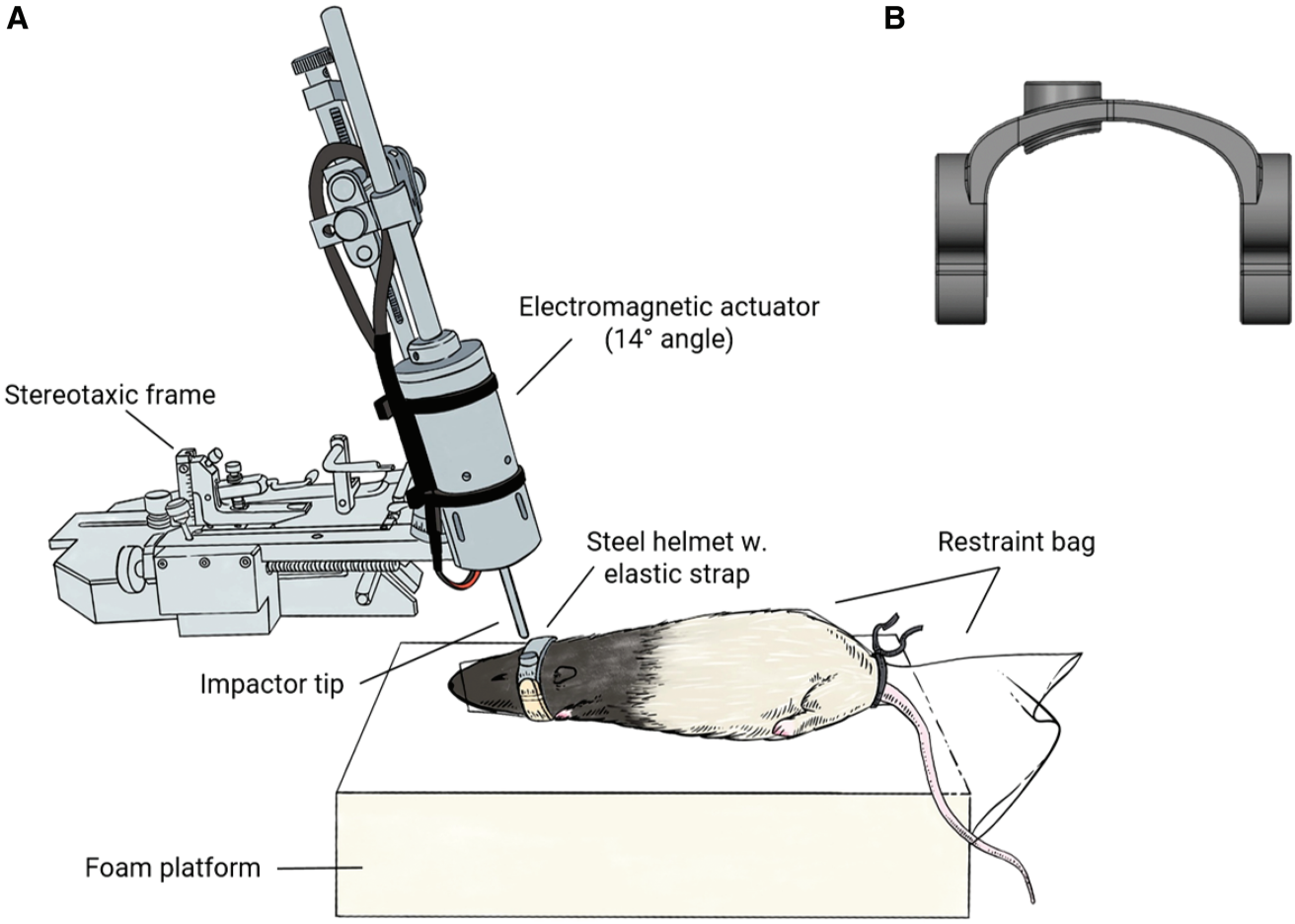

FIG 1. The awake closed-head injury model in rats. **(A)** A controlled cortical impact evice was attached to a stereotactic frame, allowing the impactor tip to be positioned at a 14° angle. The rat is placed in a cone-shaped restraint bag and fitted to the three-dimensional printed steel helmet **(B).** The helmet is secured to the head with an elastic band so that the impact target is centered over the left parietal bone. The restrained rat was placed on a foam platform and the impactor tip is centered to strike the helmet target to deliver an injury. The procedure can be completed in less than 1 min. Color image is available online.

plus maze (EPM), and Y-maze. All behavioral assessments, excluding beam walk, were recorded by an overhead camera and analyzed using tracking software Ethovision (Ethovison XT 10; Noldus, the Netherlands). In an attempt to control for any effects of restraint, rats in the 24- and 48-h recovery groups were briefly restrained at the same time interval prior to each testing task as performed for the acute injury group. All tasks were performed with paired sham and ACHI rats, with experimenters blinded to the rat groups.

Beam walk

Fine motor function was assessed at baseline, 1 min, and 24 h post-injury in a similar manner to that described previously.[36] Briefly, the apparatus consisted of a 2-cm wide, 1.5-m long wooden beam elevated 75 cm above ground, with a thin mattress placed below to soften any falls. A dark ''home box'' was placed at the end of the beam with home cage bedding inside to attract rats to complete the task. At the other end of the beam was a bright light (i.e., an aversive stimulus), as well as a camera to record the task. To complete each trial, rats were required to traverse 1.0 m to the home box. Beam training took place 3 days prior to sham/ACHI and required subjects to cross a 4-cm wide beam for five consecutive trials, followed by a further five trials on a 2-cm wide beam. Rats were gently guided across the beam during training until they each readily crossed without interference of the experimenter. For beam testing, rats were required to traverse the 2-cm wide beam for five consecutive trials before being returned to home cages. Trials

Table 1. Neurobehavioral Groups

	Group A (n = *15-16/group)*	*Group B* (n = *9-10/group)*	*Group C* (n = *9-10/group)*	*Group D* (n = *17-18/group)*
< 20 min	Beam, OF, EPM	Y-maze		
24 h	Beam	OF and EPM	Y-maze	
48 h				Y-maze

OF, open field; EPM, elevated plus maze.

began and finished when the nose of the rat crossed the start and finish line, respectively. Baseline tests were carried out on the 2 consecutive days prior to injury, with results from both days averaged to produce baseline values for each rat. In a small number of cases, trials were re-performed if rats were immobile for longer than 5 sec. Average latency to cross the beam and total number of hindlimb slips were later determined through captured video analysis by a researcher blinded to the experimental conditions.

Y-maze

Spatial memory was assessed at either 5 min, 24 h, or 48 h post-sham/ACHI using the Y-maze similar to that described previously.[37,38] Briefly, rats were introduced into a Y-maze for a 10 min exploration phase, with the novel arm closed, then returned to their home cage. One hour later, rats were reintroduced to the Y-maze for a 5-min testing phase with all arms open. For rats in the 5 min post-sham/ACHI assessment group, the exploration phase was conducted exactly 55 min prior to injury (i.e., 1 h prior to testing as for the 24 h and 48 h assessment groups). Total time spent in the novel arm, the familiar arm (expressed as mean of two familiar arms), and total distance traveled were determined.

Open field

An OF was used to assess locomotion/activity at 10 min and 24 h post-sham/ACHI similar to that described previously.[39,40] This task consisted of a 100×100 cm sawdust-covered arena, with 40 cm-high walls to prevent escape. Rats were released into the center of the field and allowed to explore for 5 min before they were returned to their home cage. Total distance traveled was determined. As a measure of anxiety, the arena was divided into a central inner zone (66×66 cm) and an outer zone and the time spent in the inner zone and was determined.

Elevated plus maze

Anxiety-like behavior and activity were assessed using an EPM similar to that described previously.[41–44] The maze is characterized by a plus-shaped platform elevated 50 cm above ground. It consists of two open and two closed arms (50×10 cm/arm) and a square (10×10 cm) central platform. Rats were placed into the center of the EPM at 15 min or 24 h post-sham/ACHI, and allowed to explore the arms of the maze for 5 min before returning to their home cage. Duration spent in the starting center zone, open arm, and closed arm, as well as distance traveled, were quantified.

Brain collection

Rats were anesthetized with Lethabarb and transcardially perfused using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before brains were removed and post-fixed with PFA at 4°C overnight. Samples were transferred into 30% sucrose solution for 4-5 days before being frozen using 2-methylbutane and dry ice and stored at -80°C.

Nissl staining

Nissl (cresyl violet) staining was used to observe morphological changes of brains collected at 1, 3, and 14 days post-sham/ACHI ($n = 3$ sham, $n = 3$ ACHI/time-point. Thirty-μm coronal sections were collected at the center of the intended impact site (bregma -4.0 mm) and mounted on slides for staining. Frozen sections were washed briefly in water before they were briefly immersed in 100% ethanol. Sections were then submerged in 100% xylene for 15 min to de-fat the tissue before they were re-immersed in 100% ethanol and rehydrated with water. Sections were stained in 0.1% cresyl fast violet for 5 min and washed in water before they were cover-slipped with DPX Mountant (Sigma, New South Wales, Australia). Images of entire sections were captured using a Leica MZ6 stereo microscope to observe any overt structural changes. Images of the cortex were captured at $100\times$ magnification using an Olympus fluorescence microscope to determine the presence of dark stained cells.

Immunofluorescence

Immunofluorescence staining was completed on 35 rat brains collected at 1, 3, and 14 days post-injury ($n = 5$-7/group at each time-point) to evaluate microglial (ionized calcium-binding adaptor molecule 1 [Iba-1]) and astrocytic (glial fibrillary acidic protein [GFAP]) density after a single ACHI. For each antibody, three coronal sections (14 μm) were collected at bregmata -3.5, -4.5, and -5.5 mm for immunofluorescence. Frozen sections were thawed and incubated in 4% PFA for 5 min before they were rehydrated with 0.01 M PBS. Sections were then blocked in 10% normal goat serum (Sigma, NSW, Australia) in PBS containing 0.2% Triton-X-100 for 1 h. Sections were incubated at 4°C overnight in primary antibodies: Iba-1 (rabbit anti-Iba-1, 1:1000; Wako USA Inc, VA) or GFAP (rabbit anti-GFAP, 1:500, Abcam; Cambridge, U.K.). Secondary antibodies for Iba-1 (Alexa Fluor 594 goat anti-rabbit, 1:500; Invitrogen, CA) and GFAP (Alexa Fluor 488 goat anti-rabbit, 1:300; Invitrogen) were applied to sections the following day for 2 h at room temperature. Sections were washed with PBS and mounted using Vectashield Mounting Medium for fluorescence containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc., CA), and a cover-slip applied before imaging. For each of the three sections per animal per antibody, captured images were centered on either the ipsilateral or contralateral parietal cortex (PCX), corpus callosum (CC), or dentate gyrus (DG) at $200\times$ magnification using an Olympus fluorescence microscope. Image J version 1.52e for Windows (National Institutes of Health, MD) was by a researcher blinded to the experimental conditions for analysis of Iba-1 and GFAP immunostaining for percentage area above threshold. Images were converted to 8-bit, and a custom threshold was chosen for each batch of staining (with paired sham and ACHI samples per time-point) to control for any inter-batch staining variation.

Statistical analysis

All outcomes were analyzed using GraphPad Prism version 7.04 for Windows (GraphPad Software, CA). All behavioral and immunofluorescence data was analyzed with two-way analysis of variance (ANOVA) and is presented as mean ± standard error of the mean. Two-way repeated measures ANOVA was used for beam task analysis only. Holm-Sidak's multiple comparisons were performed as appropriate. Statistical significance was accepted if $p < 0.05$.

Results

Sensorimotor deficits following ACHI

Sensorimotor function was assessed using the beam task at baseline, 1 min and 24 h post-injury (Fig. 2). Total number of hindlimb slips (Fig. 2A) were analyzed by two-way repeated measures ANOVA, revealing a main effect of injury ($F_{(1, 29)} = 16.83$; $p < 0.001$) and time ($F_{(2, 58)} = 21.22$; $p < 0.0001$), and a significant interaction of injury by time ($F_{(2, 58)} = 15.49$; $p < 0.0001$). *Post hoc* analysis found ACHI rats displayed significantly more hindlimb slips at 1 min post-injury compared with sham rats (Fig. 2A; $p < 0.0001$), with total number of slips resolving back to sham levels after 24 h. Notably, all 15 ACHI rats had an increased number of hindlimb slips immediately post-impact when compared with their baseline performance. There were no differences

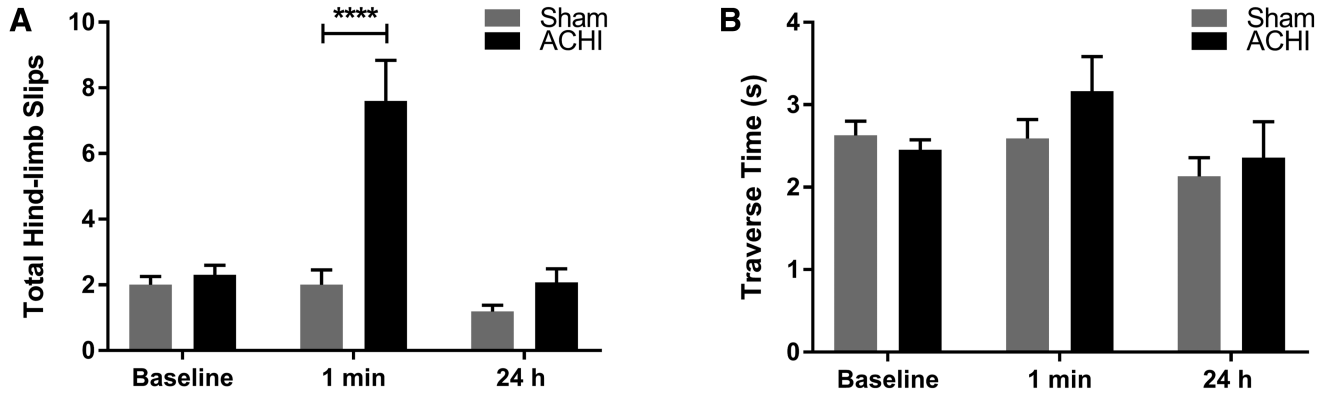

FIG 2. The effect of awake closed-head injury (ACHI) on beam task performance. **(A)** At 1 min post-ACHI, rats displayed more hindlimb slips compared with sham rats (****$p < 0.0001$), with resolution back to baseline after 24 h. **(B)** Despite the difference in footslips at the 1 min time-point, there were no differences in the time needed to traverse the beam at any time-points. $n = 15$-16/group at each time-point (repeated testing). Data presented as mean ± standard error of the mean.

between ACHI rats and sham rats in the time taken to traverse the beam at all time-points (Fig. 2B).

Impaired spatial memory post-ACHI

The effect of an ACHI on spatial memory of the Y-maze was tested at 5 min, 24 h, and 48 h post-injury in different cohorts of rats per time-point (Fig. 3A-C). At 5 min post-sham/ACHI, two-way ANOVA for time spent in the novel arm compared with the familiar arm (Fig. 3A) demonstrated a main effect of arm preference ($F_{(1, 34)} = 7.82$; $p < 0.01$), with a significant interaction of injury by arm preference ($F_{(1, 34)} = 13.12$; $p < 0.001$). Multiple comparisons revealed that at 5 min, sham rats had significant preference for the novel arm over the familiar arm when compared with ACHI rats

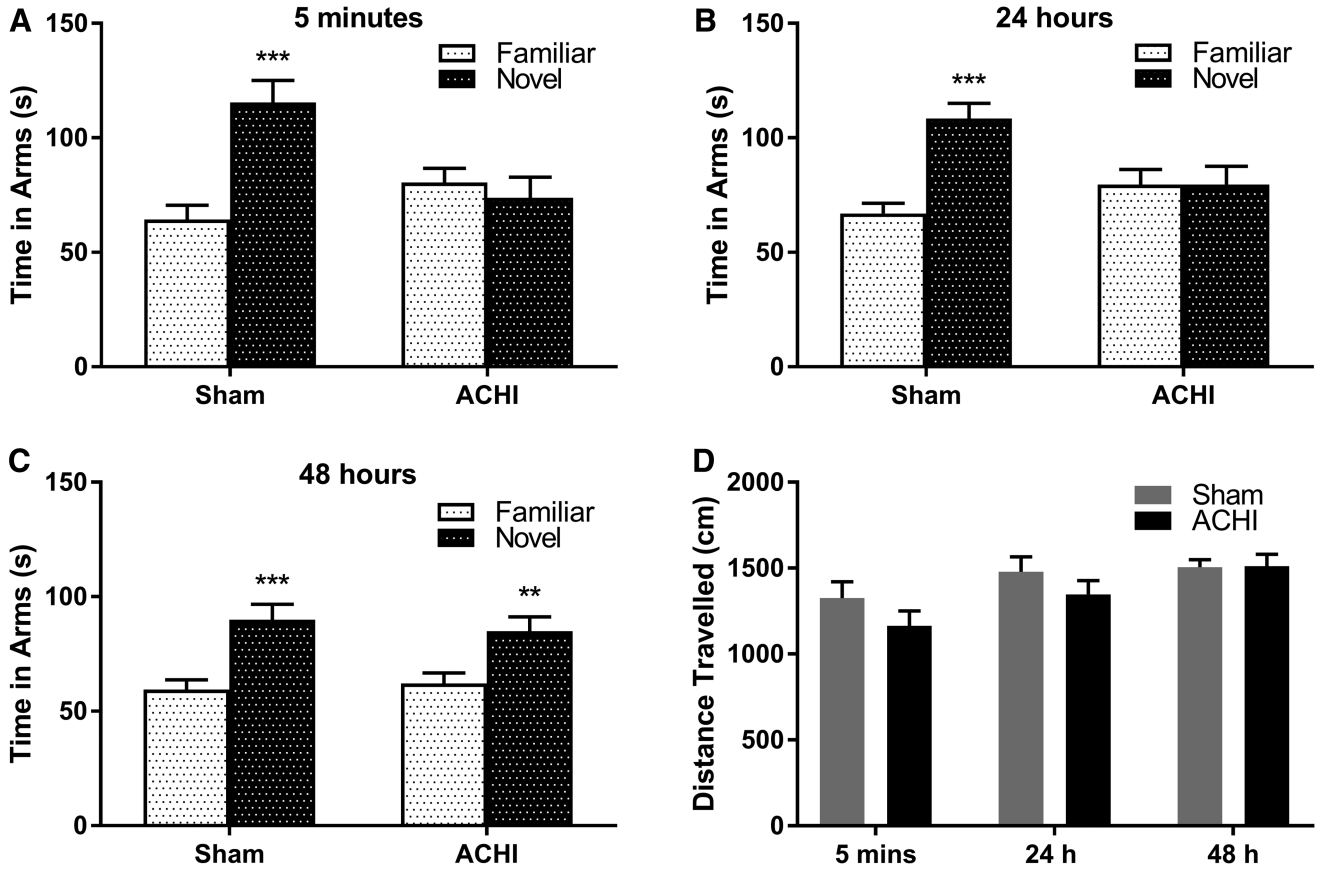

FIG 3. Y-maze performance following awake closed-head injury (ACHI). **(A)** Sham rats spent more time in the Y-maze novel arm than familiar arm when compared with ACHI rats at both 5 min (***$p < 0.001$) and **(B)** 24 h (***$p < 0.001$) post-sham/ACHI. **(C)** At 48 h, both the sham (***$p < 0.001$) and ACHI (**$p < 0.01$) groups spent more time in the novel arm compared with familiar arm. **(D)** There were no differences in distance traveled in the Y-maze between at all time-points. $n = 9$-18 per group at each time-point (no repeated testing). Data presented as mean ± standard error of the mean.

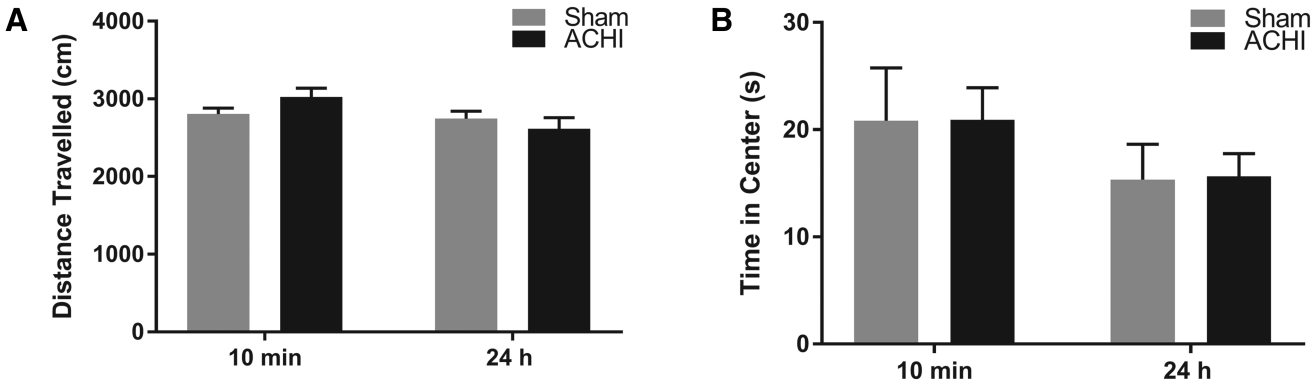

FIG 4. Distance traveled in an open field. There were no differences between sham and awake closed-head injury (ACHI) rats in **(A)** distance traveled and **(B)** time spent in the center at both 10 min and 24 h post-sham/ACHI. $n = 9$-16 per group at each time-point (no repeated testing). Data presented as mean ± standard error of the mean.

($p < 0.001$). For preference of the novel arm compared with the familiar arm at 24 h post-sham/ACHI (Fig. 3B), two-way ANOVA revealed a significant main effect of arm preference ($F_{(1, 34)} = 9.55$; $p < 0.01$) and interaction of injury by arm preference ($F_{(1, 34)} = 9.53$; $p < 0.01$). *Post hoc* comparisons found that at 24 h, sham rats had significant preference for the novel-arm over the familiar-arm compared with ACHI rats ($p < 0.001$). At 48 h, a main effect of arm preference was found (Fig. 3C; $F_{(1, 66)} = 22.91$; $p < 0.0001$), with no interaction of injury by arm preference. *Post hoc* analysis of 48 h results revealed a significant preference for the novel arm over the familiar arm in both sham ($p < 0.001$) and ACHI rats ($p < 0.01$). No differences in distance traveled were found in sham and ACHI rats across all time-points (Fig. 3D).

No differences in open field

The OF was used to observe locomotor and anxiety-like behaviors of rats at 10 min and 24 h post-sham/ACHI, with no significant differences in total distance traveled (Fig. 4A) and time spent in the inner zone (Fig. 4B) found between sham and ACHI rats.

Reduced exploratory behavior in EPM

Anxiety-like and exploratory behavior were tested using the EPM at 20 min and 24 h after sham/ACHI (Fig. 5). For the duration spent in the EPM closed arms at 20 min (Fig. 5A), two-way ANOVA showed a potential trend for a significant interaction of injury by time ($F_{(1, 46)} = 2.93$; $p = 0.09$). There were also no significant differences related to injury in time spent in the EPM open arms (Fig. 5B). For time spent in the center region of the EPM (Fig. 5C) there was an interaction of injury by time ($F_{(1, 46)} = 5.43$; $p < 0.05$), with *post hoc* comparisons revealing ACHI rats spent significantly less time in the center of the EPM compared with sham rats at 20 min ($p < 0.01$). Regarding distance traveled in the EPM, two-

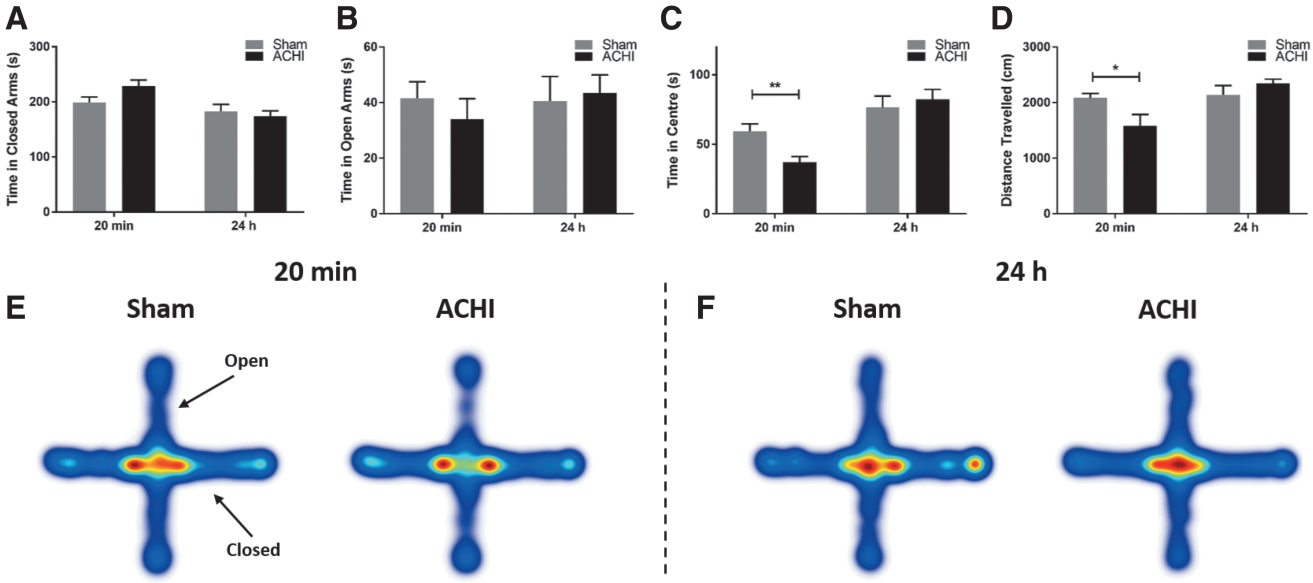

FIG 5. Effect of awake closed-head injury (ACHI) on elevated plus maze (EPM) behavior. **(A)** There was a trend for altered duration in the closed arms between injured and sham rats (injury*time interaction, $p = 0.09$). **(B)** No differences between sham and ACHI rats were found in time spent in open arms at both time-points. **(C)** ACHI rats spent less time in the center of the EPM compared with sham rats at 20 min (**$p < 0.01$), with no differences apparent at 24 h. **(D)** Distance traveled within the EPM was lower in ACHI rats compared with sham rats at 20 min (*$p = 0.05$); however, there were no such differences at 24 h. **(E)** Grouped heatmaps of sham and ACHI rats at 20 min post-ACHI depicts these subtle differences in EPM behaviors. **(F)** At 24 h, grouped heatmaps between sham and ACHI rats appear similar. $n = 9$-16 per group at each time-point (no repeated testing). Data presented as mean ± standard error of the mean. Color image is available online.

way ANOVA revealed a significant interaction of injury by time ($F_{(1, 46)} = 5.15$; $p < 0.05$), with *post hoc* comparisons revealing less distance traveled by ACHI rats when compared with sham rats at 20 min post-injury only ($p < 0.05$; Fig. 5D).

Lack of overt structural changes

Cresyl violet staining was used to visually examine for any overt structural changes at three discrete time-points following the administration of the ACHI. There was no evidence of significant structural damage at the injured ipsilateral side (Fig. 6) and no overt difference in the presence of nissl-stained dark neurons (results not shown) at 1 day, 3 days, and 14 days following the administration of the ACHI.

Microglial disturbances following ACHI

To provide insights on microglial activation, Iba-1 staining was performed on sections centered on the ipsilateral and contralateral PCX, CC, and DG of sham and ACHI brains at 1 day, 3 days, and 14 days. A main effect of injury was found for Iba-1 immunoreactivity in the ipsilateral PCX (Fig. 7A, 7G; $F_{(1, 29)} = 4.63$; $p < 0.05$); however, there was no significant injury by time interaction nor differences detected with *post hoc* comparisons. For the ipsilateral CC, there was a main effect of injury (Fig. 7B,H; $F_{(1, 29)} = 6.76$; $p < 0.05$), with *post hoc* analysis revealing increased area above threshold for Iba-1 immunostaining in the ipsilateral CC of ACHI rats when compared with sham at 3 days only (Fig. 7B, 7H; $p < 0.05$), and no such differences found at both 1 day and 14 days (Fig. 7H). There were no differences in Iba-1 labeling in the ipsilateral DG between sham and injured rats (Fig. 7C, 7I). Analysis of the contralateral PCX, CC, and DG revealed no significant effects of injury on Iba-1 staining (data not shown).

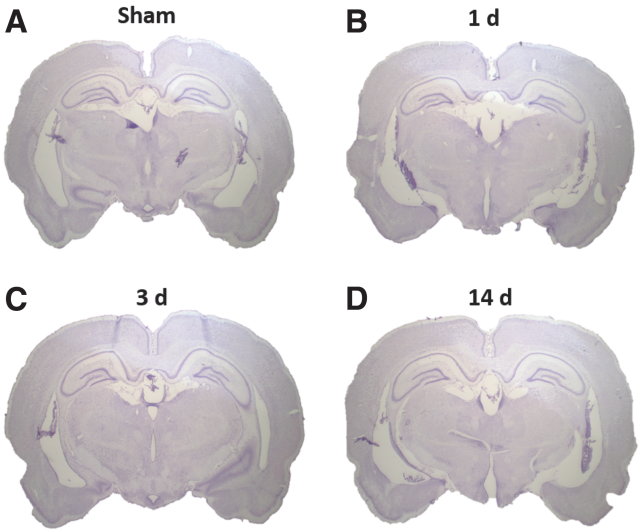

FIG 6. Nissl staining of sham and awake closed-head injury (ACHI) brains at the site of impact. Brains were collected from sham and injured rats at 1 day, 3 days, and 14 days post-sham/ACHI. Representative images of cresyl violet stained 30 μm coronal sections from **(A)** sham, **(B)** 1 day post-ACHI, **(C)** 3 days post-ACHI, and **(D)** 14 days post-ACHI. $n = 3$ per time-point. Low magnification images show no overt structural changes following ACHI (left side is ipsilateral injury site). Color image is available online.

Astrocytic changes following ACHI

Astrocyte activation was assessed using quantification of GFAP immunostaining. There were no observed differences in GFAP labeling in the ipsilateral PCX (Fig. 8A, 8G), with a potential trend for an injury and time interaction for GFAP staining in the ipsilateral CC (Fig. 8B, 8H; $p = 0.10$). However, for the ipsilateral DG, there was a significant injury by time interaction (Fig. 8C, 8I; $F_{(2, 29)} = 3.61$; $p < 0.05$), with multiple comparisons revealing a significant increase in the area above threshold for GFAP immunostaining in ACHI when compared with sham rats at 3 days post-injury ($p < 0.05$), with no such differences found at 1 day or 14 days (Fig. 8I). Analysis of the contralateral PCX, CC, and DG revealed no significant effects of injury on GFAP staining (data not shown).

Discussion

Rodent models allow highly controlled, rigorous and time-efficient investigations into the effects of concussion and the underlying mechanisms. There is a growing consensus that models that have a greater degree of face validity and minimize the use of confounds may be necessary to resolve some of the most pertinent ongoing issues in this field.[8,14] In this study, we prioritized the development of a rat model that closely replicates several features typical of clinical concussion. Our model of concussive-like injury in rats eliminates the need for anesthesia, features a controlled blow to the unrestrained head, and most importantly, consistently results in the rapid onset of impairments in neurological function that resolve within 24-48 h. The behavioral findings indicate that this model is of particular relevance to clinical concussion, where symptoms are most often relatively subtle and short-lived when compared with more severe forms of TBI. The immunohistochemical findings indicate that a single ACHI induces no overt structural changes, but results in transient and region-specific activation of microglia and astrocytes. This glial activation was found to peak at 3 days post-impact, indicating that other neurobiological mechanisms likely underlie the acute behavioral impairments.

Nature of neurobehavioral findings

Beam testing revealed that when compared with sham rats, those given a single ACHI had significant sensorimotor impairment at 1-2 min post-impact. Notably, all 15 ACHI rats had an increased number of hindlimb slips immediately post-impact when compared with their baseline performance, indicating a reliable effect of the impact on this task. Importantly, beam deficits had resolved by 24 h post-impact, suggesting that, as is often seen in clinical concussion,[7,45] sensorimotor deficits were relatively short-lived. Additional testing is required to understand the mechanisms underlying these changes, though they likely reflect temporary disturbances to one or more sensorimotor pathways, such as those involved in balance, motor control or vision. Further, although our findings suggest acute and transient changes, it is possible that more subtle sensorimotor deficits (e.g., gait alterations) may linger beyond 24 h post-impact.

Our Y-maze findings indicated that cognitive deficits also were present in the acute stages post-ACHI; however, these impairments persisted beyond the other detected behavioral changes, with differences in novel arm preference also found between sham and ACHI rats tested at 24 h post-injury. In rats tested at 48 h post-impact, a novel arm preference was found in both sham and ACHI rats, indicating no differences in spatial memory at this time-point. Taken together, these findings indicate that ACHI induced transient deficits in short-term

spatial memory, with the duration of these impairments lasting beyond the other detected behavioral changes. Though this result may represent potential differences in behavioral task sensitivity, it does align with a number of clinical studies that have reported a relative lag in cognitive recovery compared with other clinical symptoms of concussion.[7,46,47] Although the Y-maze protocol was consistent between time-points, it is important to recognize that rats in the acute testing group were exposed to the two arms of the apparatus at 1 h prior to sham/ACHI, whereas rats in the other recovery groups were firstly exposed after sham/ACHI (i.e., 1 h prior to testing at either 24 and 48 h). As such, our findings likely reflect a degree of retrograde amnesia at 5 min post-impact, and anterograde amnesia at 24 h post-impact. Further studies are required to determine the exact nature and mechanisms of these memory deficits; however, given the location of the impact site and our immunohistochemical findings, it is likely that hippocampal disturbances are involved.

We hypothesized that ACHI would result in altered anxiety-like behavior, as reported in other models of mTBI[48-50] and clinical concussion.[51,52] There were no significant differences between sham and ACHI rats in EPM arm duration and time in the center of the open field at any time-point in this study. There was, however, a potential trend (injury*time; $p = 0.09$) for injured rats to spend more time in the EPM closed arm at 20 min post-injury, as well as a significant reduction in time spent in the center zone of the EPM and total distance traveled by ACHI rats at this time-point. Although these EPM findings indicate that ACHI significantly altered behavior, it is not possible to conclude that these changes are anxiety-related, particularly given the lack of differences observed in open field testing. It is possible that anxiety-related changes may occur at other time-points post-ACHI. Further, it is also possible that beam and open field testing prior to EPM confounded these results; however, some laboratories have reported minimal effects of behavioral test batteries on EPM performance,[53] and in fact, there is some evidence that pre-exposure to a novel environment (e.g., open field) shortly before EPM testing increases motor activity and the likelihood of entering the EPM open arms,[53,54] potentially increasing the likelihood of detecting anxiolytic behavior. Another factor that may limit interpretation of the anxiety-related behavior in the EPM is our finding of reduced distance traveled in the EPM by ACHI rats at 20 min post-injury.

Although there were no differences in distance traveled in the Y-maze or open field at 5 min and 10 min, respectively, the significant reduction in distance traveled by ACHI when compared with sham rats in the EPM at 20 min post-impact is a point of interest. The contrasting findings in distance traveled between these tasks likely reflects either a time- or task-dependent effect of injury on locomotor activity. It is possible that several symptoms induced by ACHI contributed to the reduced exploration of the EPM, including

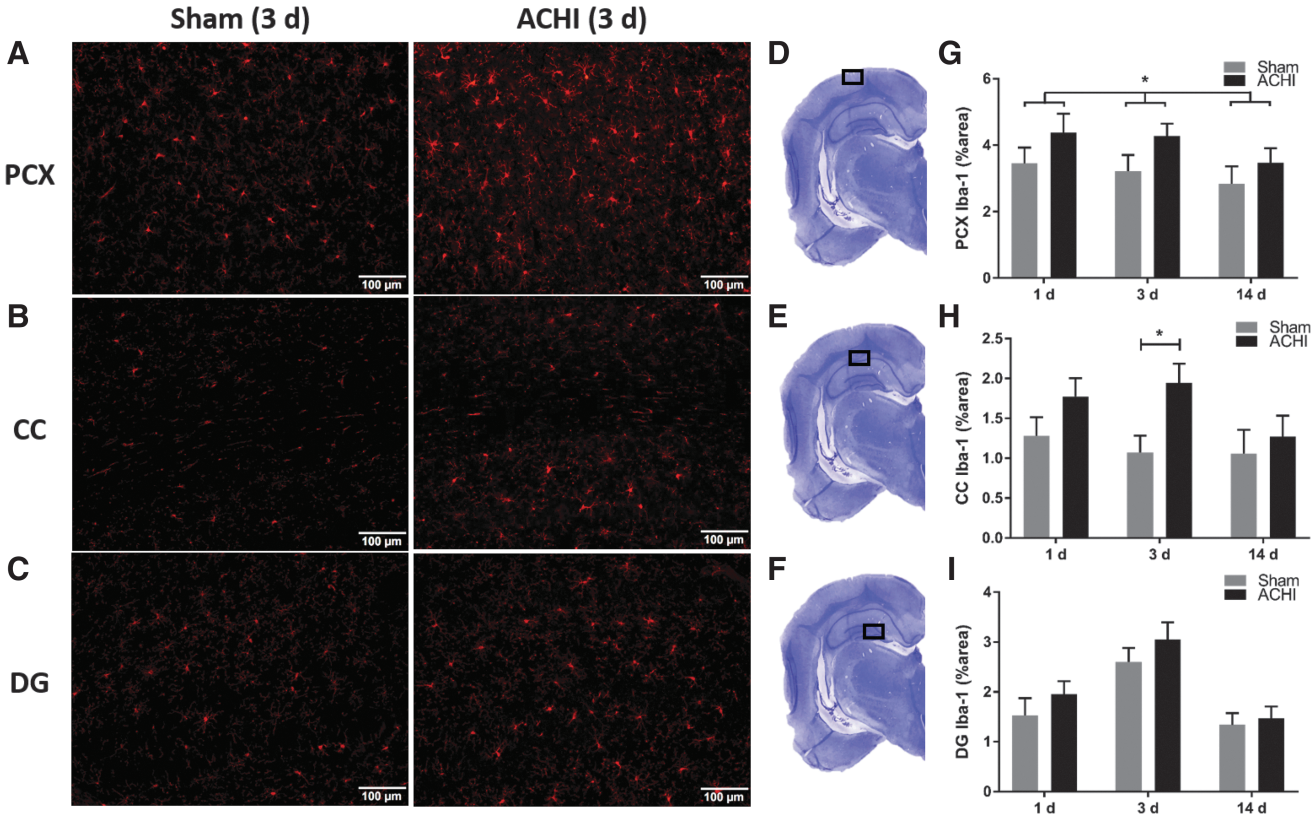

FIG 7. Effect of awake closed-head injury (ACHI) on microglial reactivity. Brains were collected at 1 day, 3 days, and 14 days after single sham or ACHI procedure for quantification of ionized calcium-binding adaptor molecule 1 (Iba-1) immunoreactivity. Images of three regions per sample were taken at 200× magnification. Representative images of Iba-1 immunofluorescence staining in the ipsilateral **(A)** parietal cortex (PCX), **(B)** corpus callosum (CC), and **(C)** dentate gyrus (DG) of sham and single ACHI rats 3 days post-procedure. Images were centered over **(D)** PCX, **(E)** CC, and **(F)** DG. **(G)** There was an overall effect of injury on Iba-1 immunoreactivity in the ipsilateral PCX (*$p < 0.05$). **(H)** Percentage area of Iba-1 also was increased in the CC of ACHI rats compared with sham rats at 3 days post-injury (*$p < 0.05$), with no differences between groups at 1 day and 14 days. **(I)** There were no differences in Iba-1 labeling in the ipsilateral DG. $n = 5$-7 per group at each time-point. Data presented as mean ± standard error of the mean. Color image is available online.

motor impairments, fatigue, drowsiness, or nausea. Regardless of the underlying mechanisms, this finding further demonstrates that a single ACHI induced transient neurobehavioral disturbances.

The use of awake animals during the injury procedure has the potential to increase heterogeneity within the model. The ACHI model, as with other closed-head models, is likely to have some degree of variability greater than that of TBI models involving craniotomy i.e. fluid percussion injury and CCI. With that said, the use of the CCI device allows for precise control of parameters that can be inconsistent in weight-drop models, and significant care was taken to ensure consistent positioning of the fitted helmet for each rat. Importantly, the aforementioned neurobehavioral findings, when considered alongside with our glial reactivity data, indicate that our protocol resulted in relatively consistent injury responses between animals.

There are a number of potential areas of interest for future studies using the ACHI model. First, although the observed sensorimotor and cognitive deficits resolved within 24-48 h, it is possible that other deficits may have developed later. Therefore, future studies should investigate longer recovery times prior to behavioral testing. Second, although we chose to use a fitted steel helmet to allow a targeted, perpendicular and diffuse blow to be delivered without skull fracture, it is possible that in similar manner to Jamnia and colleagues,[20] the impact tip could be positioned so as to directly strike the rodent head. As such, future studies may investigate the impact and necessity of the fitted helmet. In addition, our experiments were designed to replicate features of clinical concussion (i.e., transient neurological symptoms induced by an impact to an un-restricted head). However, detailed characterization of head kinematics, such as that described for CHIMERA,[22,55] is required to determine how this model reflects the biomechanics typical of concussion.

Finally, although a variety of measures were taken to minimize the extent of animal distress and discomfort (e.g., acclimatization protocol, restraint scoring, short duration of sham/injury procedure), we acknowledge that the necessity for awake animal use is ultimately dependent on the impact of anesthesia on the outcomes of interest. Although there are several lines of evidence that anesthetics can alter neuropathological and neurobehavioral outcomes, further studies are required to determine the influence of a brief period of anesthesia in this model.

Transient, region-specific glial reactivity following ACHI

Immunofluorescence staining for Iba-1 and GFAP revealed region-specific increases in glial reactivity ipsilateral to the impact site, peaking at 3 days. Increases in labeling for microglial/macrophage marker Iba-1 were most pronounced around the CC, whereas astrocytic marker GFAP was found to increase in the DG. These findings likely reflect an increase in reactive microglia and

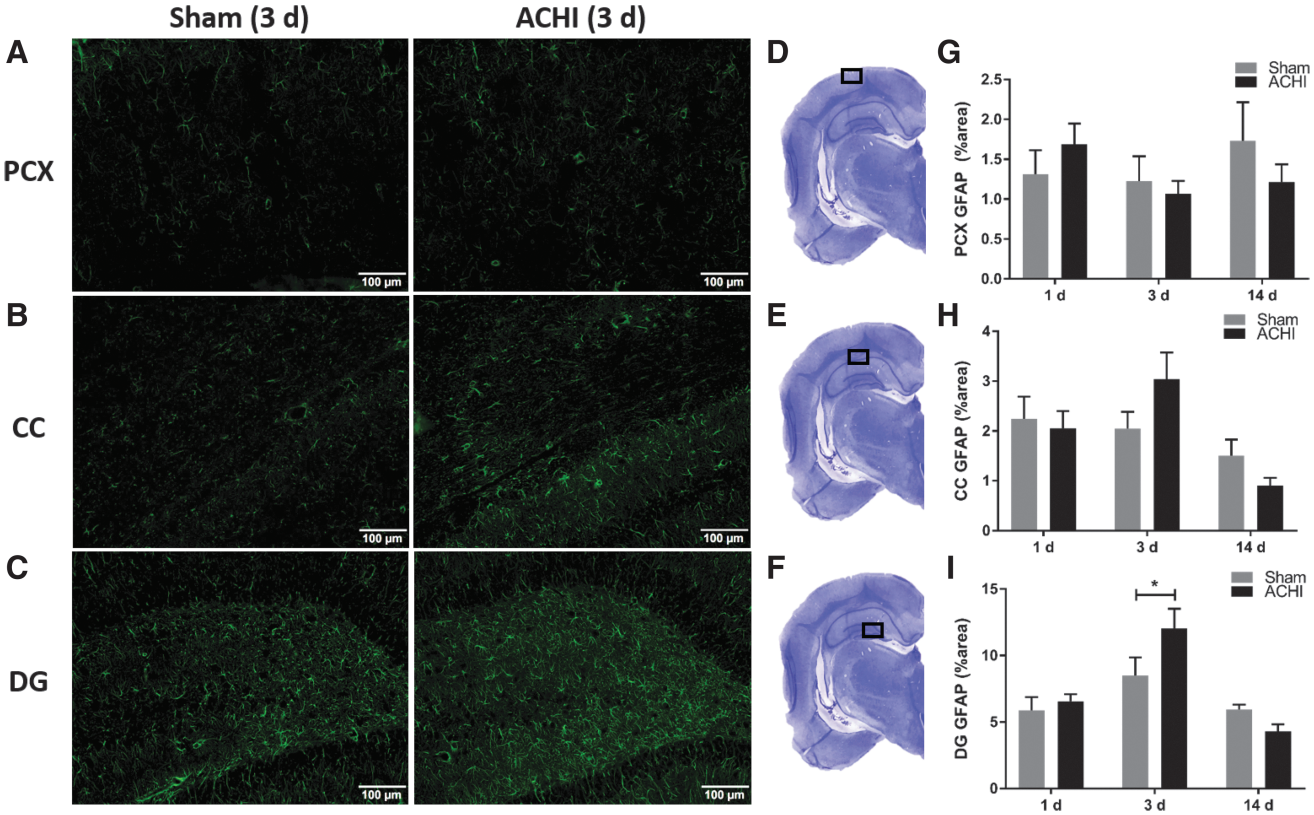

FIG 8. Effect of awake closed-head injury (ACHI) on astrocytic reactivity. Brains were collected at 1 day, 3 days, and 14 days after single ACHI or sham procedure for quantification of glial fibrillary acidic protein (GFAP) expression. Images of three regions per sample were taken at 200×magnification. Representative images of GFAP immunofluorescence staining in the ipsilateral **(A)** parietal cortex (PCX), **(B)** corpus callosum (CC), and **(C)** dentate gyrus (DG) of sham and single ACHI rats 3 days post-procedure. Images were centered over **(D)** PCX, **(E)** CC, and **(F)** DG. There was no significant effect of ACHI on GFAP expression in the PCX **(G)** and CC **(H). (I)** GFAP labeling was increased in the DG for ACHI rats compared sham rats at 3 days (*$p < 0.05$), with no differences found at 1 and 14 days. $n = 5$-7 group at each time-point. Data presented as mean± standard error of the mean. Color image is available online.

astrocytes.[56–59] Interestingly, we observed no differences in expression of these glial reactivity markers at 1 day post-ACHI, indicating that the persistent Y-maze deficits we observed at this time-point were likely due to other neurobiological changes induced by impact. In addition, our findings of neurobehavioral resolution prior to the detected peak in glial reactivity adds to the growing evidence that symptom resolution may precede neurobiological recovery from concussive injury.[60,61] Future studies will investigate how this seemingly delayed-onset but transient increase in glial reactivity may contribute to the potential temporal window of increased cerebral vulnerability to exacerbated or persistent effects of repeated concussions. Despite these promising findings, we acknowledge that futures studies incorporating more comprehensive histological techniques (e.g. unbiased stereology) are required to create a greater understanding of the timing and nature of the glial effects induced by this model.

Conclusion

This study investigated the temporal changes in neurobehavior and glial reactivity induced by a novel model of concussive-like injury in conscious rats. The elimination of anesthetic and surgical procedures in our high-throughput ACHI model not only removes the potential confounds these factors may have on pathophysiology, but also significantly allowed for detailed neurobehavioral assessments to be made within 20 min of the impact. Importantly, the findings from this study demonstrated that a single ACHI induced the rapid onset of neurobehavioral impairments, all of which had resolved within 24-48 h. Our immunohistochemical findings indicate that glial reactivity peaked at 3 days post-impact, notably after the time-point of neurobehavioral resolution.

Such temporal characterization of the effects of a single mTBI is often overlooked, but is of particular importance when attempting to mimic concussion in humans. In particular, although a number of recent rodent-based studies have demonstrated cumulative and potentially chronic effects of repeated concussive-like injuries, the clinical relevance of repeated impacts delivered prior to a defined time-point of symptom resolution is questionable, particularly given the recent improvements in clinical concussion management (e.g., return to play guidelines). We postulate that prioritization of the clinical relevance of both the animal model and study design is necessary to increase our understanding of the neurobiological basis and effects of concussions, and as such, to provide new insights that may lead to improved clinical management.

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Author Disclosure Statement

No competing financial interests exist.

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Appendix E: Published Chapter 3

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Behavioral, axonal, and proteomic alterations following repeated mild traumatic brain injury: Novel insights using a clinically relevant rat model

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ABSTRACT

A history of mild traumatic brain injury (mTBI) is linked to a number of chronic neurological conditions, however there is still much unknown about the underlying mechanisms. To provide new insights, this study used a clinically relevant model of repeated mTBI in rats to characterize the acute and chronic neuropathological and neurobehavioral consequences of these injuries. Rats were given four sham-injuries or four mTBIs and allocated to 7-day or 3.5-months post-injury recovery groups. Behavioral analysis assessed sensorimotor function, locomotion, anxiety, and spatial memory. Neuropathological analysis included serum quantification of neurofilament light (NfL), mass spectrometry of the hippocampal proteome, and ex vivo magnetic resonance imaging (MRI). Repeated mTBI rats had evidence of acute cognitive deficits and prolonged sensorimotor impairments. Serum NfL was elevated at 7 days post injury, with levels correlating with sensorimotor deficits; however, no NfL differences were observed at 3.5 months. Several hippocampal proteins were altered by repeated mTBI, including those associated with energy metabolism, neuroinflammation, and impaired neurogenic capacity. Diffusion MRI analysis at 3.5 months found widespread reductions in white matter integrity. Taken together, these findings provide novel insights into the nature and progression of repeated mTBI neuropathology that may underlie lingering or chronic neurobehavioral deficits.

1. Introduction

Mild traumatic brain injuries (mTBIs), such as concussions, account for >80% of TBI cases (Dewan et al., 2018). mTBI is particularly common in sports with the highest participation rates for adolescents and young adults (Daneshvar et al., 2011; Finch et al., 2013). It is also increasingly prevalent in military personnel (e.g. warzone blast exposure (Escolas et al., 2020; Lindquist et al., 2017)), and is a leading cause

Abbreviations: mTBI, mild traumatic brain injury; NfL, neurofilament light; MRI, magnetic resonance imaging; PPCS, persistent post-concussion symptoms; CTE, chronic traumatic encephalopathy; ACHI, awake closed head injury; DTI, diffusion tensor imaging; PND, post-natal day; EPM, elevated plus maze; NE, north-east; NW, north-west; SE, south-east; SW, south-west; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TCEP, tris(2-carboxyethyl)-phosphine-hydrochloride; CAA, 2-Chloroacetamide; FA, fractional anisotropy; AD, axial diffusivity; RD, radial diffusivity; ADC, apparent diffusion coefficient; TBSS, tract-based spatial statistics; TFCE, threshold free cluster enhancement; ROI, region of interest; ANOVA, analysis of variance; CC, corpus callosum; EC, external capsule; IC, internal capsule; Fim, fimbria of hippocampus.

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of emergency department visits (Centers for Disease Control, 2019; Gaw and Zonfrillo, 2016). Although the neurobehavioral (e.g. cognitive and emotional impairments) and physical (e.g., headache, motor deficits) effects of mTBI most often resolve within 7–10 days, approximately 20% of individuals are plagued by symptoms that persist for several weeks, months or longer (i.e. persistent post-concussion symptoms; PPCS) (Quinn et al., 2018). There is mounting evidence that a history of mTBI may be the strongest risk factor for suffering a future mTBI, and also for experiencing more symptoms for a longer duration should another mTBI occur (Dretsch et al., 2015; Iverson et al., 2017; Miller et al., 2013). Risk of exacerbated symptoms is thought to be particularly high if mTBIs are experienced in short succession (Eisenberg et al., 2013; Silverberg et al., 2013). Moreover, several recent retrospective studies on individuals with a history of mTBI or repetitive head trauma have provided evidence of an increased risk for long-term neurological conditions, such as depression and cognitive impairment (Manley et al., 2017), or progressive neurodegenerative diseases such as Alzheimer's and chronic traumatic encephalopathy (CTE) (Lehman et al., 2012; Mez et al., 2017). However, there is still a great deal of controversy and debate surrounding the associations between repeated mTBI and persistent or progressive neurological conditions (Iverson et al., 2019; Smith et al., 2019), and this topic requires further investigation.

The last two decades have seen a significant increase in understanding of the neuropathology that may underlie the effects of repeated mTBIs. Post-mortem analyses of individuals with an extensive history of mTBI have found evidence of microstructural pathology, including widespread axonal disruption and proteopathies, and in some cases, significant atrophy of certain brain regions (McKee et al., 2013; Smith et al., 2013). Clinical in vivo neuroimaging studies have also provided evidence of reduced white matter integrity (Koerte et al., 2015; Wright et al., 2020), tau pathology (Barrio et al., 2015; Cherry et al., 2016) and microglial activation in people with a history of repeated mTBI (Cherry et al., 2016; Coughlin et al., 2015). Nonetheless, the retrospective nature and presence of confounding variables in many of these studies make it difficult to decipher the true prevalence and magnitude of these neuropathological changes. Moreover, with most studies conducted at single time-points several years or decades following the final injury, the temporal progression of neuropathology is poorly understood.

Animal models of repeated mTBI have allowed for important neuropathological insights not possible in clinical studies. Multiple rodent studies have found that repeated mTBIs can trigger significant and potentially lasting changes in energy metabolism, blood brain barrier damage, oxidative stress, neuroinflammation, axonal damage, tau pathology, and neurodegeneration (Cheng et al., 2019; Eyolfson et al., 2020; Fehily and Fitzgerald, 2017; Lyons et al., 2018; Mouzon et al., 2018; Shultz et al., 2011; Webster et al., 2015; Wright et al., 2019). Nevertheless, the clinical relevance of some animal models used to create this understanding has been questioned, with anaesthetics or craniotomy both shown to alter TBI pathophysiology and outcomes, and some models producing structural changes (e.g. cavitation) not typically seen in clinical mTBI (Archer et al., 2018; Shultz et al., 2017). Moreover, although clinical and pre-clinical studies have increased understanding of the potential cellular and molecular consequences of repeated mTBI, a number of important questions remain, particularly regarding the progression of these changes through the acute and chronic stages of injury. To provide further insights into the pathophysiological and neurobehavioral effects of mTBIs, we have recently developed and characterized an awake, closed-head injury (ACHI) model in rats. A single injury with this anaesthetic- and surgery-free model results in transient neurobehavioral symptoms that typically resolve within 24–48 h post-injury, and transient increases in glial reactivity that appear to resolve within two weeks (Pham et al., 2019). We have also recently shown that repeated ACHIs result in acute changes in white matter that resemble those seen in clinical mTBI (Wortman et al., 2018). In the current study, we aimed to determine the acute and chronic effects of repeated ACHIs separated by 48 h. To do so, we implemented a detailed neurobehavioral battery to characterize the extent and evolution of neurobehavioral changes, blood (neurofilament light; NfL) and neuroimaging (diffusion tensor imaging; DTI) biomarkers to assess axonal injury, and high-resolution mass spectrometry to investigate the hippocampal proteome at both acute and chronic stages after repeated mTBI.

2. Materials and methods

2.1. Animals

Sixty-four male Long-Evans rats were obtained from Animal Resource Centre (WA, Australia) at post-natal day (PND) 28. Rats were housed in pairs at the La Trobe University Central Animal House, with a 12 h:12 h light/dark cycle and food and water available ad libitum. All procedures were performed in accordance with the Animal Ethics Committee at La Trobe University (AEC 17–06) and were within the guidelines of the Australia code of practice for the use of animals for scientific purposes by the Australian National Health and Medical Research Council. All animals were allowed 48 h to acclimatize to their enclosures upon arrival before experimenter contact. After this period animals were handled 4 days per week until the first sham or injury procedure on PND 40–42.

2.2. Mild TBI

Rats were randomly allocated into groups that received four sham injuries or four ACHI procedures, with each procedure separated by 48 h. This injury interval was chosen as we previously found behavioral deficits at 48 h post-ACHI (Pham et al., 2019). Sham and ACHI procedures were completed similar to those described previously (Pham et al., 2019). Briefly, a steel helmet was placed over the Decapicone™-restrained rat (Braintree Scientific, MA, United States) and centered over the left parietal bone, with the rat placed on a foam bed and lightly held in position by the lower body. Once positioned, a 5 mm tip attached to a controlled cortical impactor (Leica Biosystems, IL, United States) was triggered and impacted the helmet (velocity: 6.5 m/s, depth: 8 mm, dwell time: 100 ms). The total duration of the procedure was less than 1 min. Sham procedures were performed identically to ACHIs, with the impact instead triggered adjacent to the animal's head. No skull fractures were observed during post-mortem analysis. The lack of anesthesia allowed for inspection of visual signs that may be associated with loss of consciousness (e.g. apnea, absence of hindlimb withdrawal reflex, lying motionless). Incidence of apparent transient loss of consciousness increased after each ACHI (1st ACHI, 25.8%; 2nd ACHI, 35.5%; 3rd ACHI, 45.2%) before slightly decreasing at the 4th ACHI (29.0%). Although these measures were conducted by an experimenter blinded to the experimental condition, it should be acknowledged that assessment of loss of consciousness is inevitably a subjective measure in this context. Once rats had self-righted and were mobile (typically within 1 min post-injury), the beam task was used to assess sensorimotor coordination for all rats for each procedure (as described below). One rat was excluded prior to injury due to a skin wound sustained in its home cage. Rats were assigned into either acute 7-day or chronic 3.5-month recovery groups.

2.3. Neurobehavioral testing

All rats performed beam task immediately post-each injury. Rats assigned to seven-day recovery (i.e. acute) performed open field (24 h following final sham/ACHI), Y-maze (two days), elevated plus maze (EPM; three days) and beam task (three days). To avoid repeated testing, rats assigned to chronic recovery were exposed to the same tasks beginning at three months, with the addition of water maze. All behavioral assessments, excluding beam task (recorded with a stationary camera), were recorded by an overhead camera and analyzed using tracking software Ethovision (Ethovision XT 10; Noldus, the Netherlands). With the exception of the beam task conducted

immediately post-injury, experimenters were blinded to the rat group at all stages during testing. All video/data analysis was conducted by a researcher blinded to the experimental conditions.

Sensorimotor function was assessed via the beam task at baseline one hour prior to the first injury, and one minute post each procedure for all recovery groups similar to that described previously (Pham et al., 2019; Wright et al., 2016). The acute recovery rats repeated the task at three days following the final sham/ACHI, and chronic rats 3.5 months. The task consisted of a 2 cm wide, 1.5 m long wooden beam, elevated 75 cm above the ground, with a protective mattress placed below to soften any falls. At the start-end of the beam there was a bright light (i.e. an aversive stimulus), as well as a camera to record the task. At the finish-end, a dark "home box" was placed with home cage bedding to encourage completion of the task. Prior to testing, beam training took place as previously described (Pham et al., 2019). Rats were required to traverse 1.0 m on the beam to the home box to complete a trial, with five trials used for each session. In a small number of cases, trials were re-performed if rats were immobile for longer than five seconds. Average latency to cross the beam and hindlimb slips were later determined through captured video analysis by a researcher blinded to the experimental conditions. Rats that failed to complete a trial within 20 s were assigned this value. Rats that fell were automatically given this maximum time as well as 2 hindlimb slips.

Locomotion/activity was assessed in an open field similar to that described previously (Brady et al., 2015; Pham et al., 2019). This task consisted of a 100 × 100 cm sawdust-covered arena, with 40 cm high walls to prevent escape. Rats began in the center of the field and were allowed to explore for five minutes before they were returned to their home cage. Locomotion was assessed by total distance travelled. To measure anxiety, the arena was divided into an inner zone (66 × 66 cm^2) and outer zone and the time spent in the inner zone was determined.

We previously found that a single ACHI induced spatial memory deficits in the Y-maze at 24 h, with resolution apparent at 48 h (Pham et al., 2019). Therefore, the current study used Y-maze to assess spatial memory, similar to that described previously (Johnstone et al., 2018; Pham et al., 2019), 48 h after the final injury in the acute recovery groups, as well as at 3.5 months in the chronic recovery groups. Rats were introduced into a Y-maze for a 10 min exploration phase, with the novel arm closed, then returned to their home cage. One hour later, rats were reintroduced to the Y-maze for a 5 min testing phase with all arms open. Entries and time spent in the novel arm and familiar arm were recorded and used to generate a discrimination preference index for each measure. The discrimination index was calculated as follows: (novel arm - familiar arm) / (novel arm + familiar arm) (Rachmany et al., 2013).

Anxiety-like behavior was measured using an EPM similar to that described previously (Pham et al., 2019; Sun et al., 2019). The plus-shaped maze platform was elevated 50 cm above ground and consisted of two open and two closed arms (50 × 10 cm/arm) and a square (10 × 10 cm) central platform. Rats were placed into the center of the EPM and allowed to explore the arms of the maze for 5 min before returning to their home cage. Duration spent in the open arms was quantified.

After the completion of other behavioral tasks, spatial cognition of chronic recovery rats was assessed using water maze similar to as previously described (Webster et al., 2015). A 163 cm diameter circular pool was filled with tap water (28 ± 1 °C). Non-toxic white paint (150 ml) was used to opacify the water and create contrast for rat tracking. The arena was divided into four quadrants, north-east (NE), north-west (NW), south-east (SE) and south-west (SW). A circular 10 cm diameter acrylic escape platform was submerged 2 cm below the water surface in the center of a randomized quadrant. Testing consisted of two days; acquisition (day 1) and reversal (day 2) consisting of 10 trials per day with a maximum time of 60 s per trial. Trials began when rats were placed into the pool facing the wall at one of eight pseudorandomized locations (N, S, E, W, NE, NW, SE, SW) and required to swim to locate and stand on the hidden platform to be 'rescued' by the experimenter. If the maximum time elapsed, rats were led to the hidden platform by the experimenter and left for 15 s before being 'rescued'. Settings for reversal were the same as for acquisition, with the exception of the hidden platform, which was placed in the opposite quadrant before trials commenced. Average latency to platform, percentage of time in target quadrants and percentage of direct and circle swims were calculated.

2.4. Tissue and blood collection

Rats were euthanized for collection of fresh or fixed tissue at either seven days post- or 3.5 months post-last injury. To obtain fresh tissue, rats were anesthetized with Lethobarb and culled for collection. The ipsilateral cortex and hippocampus were flash frozen in liquid nitrogen and stored at −80 °C before analysis. Blood was collected by cardiac puncture and transferred to 3.5 ml Becton Dickinson Vacutainer® SST™ II Advance blood collection tubes (Franklin Lakes, NJ, United States), and allowed to clot at room temperature for 30 min. Following centrifugation at 1500*g* for 10 min, serum was snap frozen in liquid nitrogen and stored at −80 °C until analysis. Chronic recovery rats were anesthetized with Lethobarb, and following cardiac blood collection, were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS before brains were removed and post fixed with PFA at 4 °C overnight. Samples were then transferred to 1× PBS and washed twice daily for 3 days on a rocker at 4 °C and stored in 1× PBS at 4 °C before they were prepared for magnetic resonance imaging (MRI).

2.5. Serum NfL quantification

Quantification of serum NfL was performed using a 'Simoa® NF-light Advantage Kit' run on the Simoa HD-X Analyzer (Quanterix, Billerica, MA, USA). A single assay was performed on eight randomly selected samples per group, and was run in a temperature-controlled laboratory by an experimenter blinded to the experimental conditions. Samples were tested in duplicate, with a total serum volume for each sample of 106 µl. All samples measured above the lower limit of quantification for NfL (0.174 pg/ml).

2.6. High-resolution, data-independent acquisition mass spectroscopy

The ipsilateral hippocampus was chosen for proteomic analysis, as our previous study using this ACHI model found evidence of hippocampal gliosis (Pham et al., 2019). Flash frozen ipsilateral hippocampus samples were homogenized in liquid nitrogen and lysed in 4% SDS, 100 mM HEPES, pH 8.5, which was then heated at 95 °C for 5 min and sonicated three times for 10 s each at an amplitude of 10 µm. The lysates were clarified by centrifugation at 16,000*g* for 10 min and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo). Equal protein amounts were denatured and alkylated using Tris(2-carboxyethyl)-phosphine-hydrochloride (TCEP) and 2-Chloroacetamide (CAA) at a final concentration of 10 mM and 40 mM, respectively, and incubated at 95 °C for 5 min. SDS was subsequently removed by chloroform/methanol precipitation. Sequencing grade trypsin was added at an enzyme to protein ratio of 1:100 and the reaction was incubated overnight at 37 °C. The digestion reaction was stopped by adding formic acid to a concentration of 1%. The samples were cleaned up with BondElut Omix Tips (Agilent) and concentrated in a vacuum concentrator prior to analysis by mass spectrometry.

Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap column (100 µm × 2 cm, nanoViper, C18, 5 µm, 100 Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 µm × 50 cm, nanoViper, C18, 2 µm, 100 Å; Thermo Scientific). The peptides were separated by increasing concentrations of 80% ACN / 0.1% FA at a flow of 250 nl/min for 158 min and analyzed with a

QExactive HF mass spectrometer (Thermo Scientific) operated in data-independent acquisition (DIA) mode. Sixty sequential DIA windows with an isolation width of 10 *m/z* have been acquired between 375 and 975 m/z (resolution: 15.000; AGC target: 2e5; maximum IT: 9 ms; HCD Collision energy: 27%) following a full ms1 scan (resolution: 60.000; AGC target: 3e6; maximum IT: 54 ms; scan range: 375–1575 m/z).

2.7. Mass spectrometric data analysis

The acquired DIA data have been evaluated in Spectronaut 13 Laika (Biognosys) using an in-house generated spectral library derived from rat brain samples. Of note, potential plasma protein contaminants have been removed from this library using an in-house generated spectral library derived from rat plasma samples in combination with homologs identified in the Human Blood Atlas (Uhlen et al., 2019). Missing values have been imputed using background signals, protein intensities were $\log_2$ transformed and median normalized, and two pairwise comparisons (7 day ACHI vs. 7 day sham and 3.5 months ACHI vs. 3.5 months sham) were carried out. Protein fold changes and FDR adjusted *p*-values (q-values) were calculated. GO terms associated with differentially regulated proteins (i.e. those with q-value <0.05 were extracted from String database (Szklarczyk et al., 2019).

2.8. DTI

2.8.1. Ex vivo magnetic resonance imaging acquisition

Ex vivo MRI was performed using a 9.4 T Bruker (Bruker™ BioSpin®, USA) and actively decoupled volume transmit and four-channel cryogenically-cooled surface receive coils. Whole fixated brains were embedded with 3% agar in 50 ml Falcon tubes for scanning. DTI was performed in 81 directions with diffusion duration (δ) = 4.5 ms, diffusion separation (Δ) = 13.5 ms and b-value = 4000 s/mm^2. We also obtained one non-diffusion (b_0) volume. Image parameters also included repetition time (4500 ms), echo time (34 ms), field of view (32 × 24 mm^2), 48 slices of 250 μm thickness and matrix size (128 × 96) giving an isotropic spatial resolution of 250 × 250 × 250 μm^3.

2.8.2. MRI processing and analysis

MRtrix3 (www.mrtrix.org) was used to process DTI (Tournier et al., 2019). DTI metrics including fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD) and the apparent diffusion coefficient (ADC) were calculated and registered to a study-specific template as described previously (Wright et al., 2018). Whole-brain voxel-wise analysis was performed using tract-based spatial statistics (TBSS) with threshold free cluster enhancement (TFCE).

ITK-SNAP (Yushkevich et al., 2006) was used to manually trace regions of interest (ROI) on seven consecutive coronal MRI T_2-weighted structural images. ROIs included the cortex, corpus callosum and hippocampus and the total volume of each ROI was calculated using ITK-SNAP.

2.8.3. Statistical analysis

All outcomes were analyzed using GraphPad Prism version 8.3.1 for Windows (GraphPad Software, CA). Water maze percentage time in target quadrant, direct and circle swims were analyzed using unpaired *t*-test and presented as mean + standard error of the mean. For correlation analysis of serum NfL levels and beam performance, Pearson r was used to assess normally distributed data, while Spearman r was used to assess data not normally distributed. Permutation testing of DTI metrics was performed using FSL's *randomize* with 5000 permutations and fully corrected for multiple comparisons with TFCE. Two-way repeated measures analysis of variance (ANOVA) were used for beam task analysis immediately after injury. All other data was analyzed with two-way ANOVA, with variables of injury and time. Sidak's multiple comparisons performed where appropriate. Statistical significance was accepted if $p \leq 0.05$.

3. Results

3.1. ACHI resulted in sensorimotor deficits immediately post-injury

Beam task was used to assess sensorimotor function at baseline and one minute after each sham/injury procedure (n = 31/group; Fig. 1). The average time taken to traverse the beam one minute after each ACHI (Fig. 1a) was analyzed using repeated measures two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)} = 60.27$; $p < 0.0001$) and time ($F_{(4, 240)} = 8.543$; $p < 0.0001$), and a significant interaction of injury by time ($F_{(4, 240)} = 13.88$; $p < 0.0001$). Post hoc analysis found repeated ACHI rats took significantly longer to traverse the beam compared to sham rats after each ACHI procedure (Fig. 1a; $p < 0.0001$ for all time-points). Total number of hindlimb slips 1 min after each ACHI (Fig. 1b) was analyzed using two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)} = 114.0$; $p < 0.0001$) and time ($F_{(4, 240)} = 18.74$; $p < 0.0001$), and a significant interaction of injury by time ($F_{(4, 240)} = 18.51$; $p < 0.0001$). Post hoc comparisons found that injured rats displayed significantly more hindlimb slips at 1 min after each ACHI compared to sham rats (Fig. 1a; $p < 0.0001$ for all time-points).

3.2. Repeated ACHI resulted in persistent sensorimotor deficits

Beam task was also used to assess sensorimotor deficits at three days (n = 14-15/group) and 3.5 months (n = 17/group) after the final injury (Fig. 2). One rat, allocated to the long-term recovery group, completed beam task at 3 days and therefore was included for this analysis only. For time taken to traverse the beam three days and 3.5 months after final-sham/ACHI (Fig. 2a), a two-way ANOVA was performed after excluding one rat with incomplete data (video recording error), and revealed a main effect of injury ($F_{(1, 59)} = 6.44$; $p = 0.014$), and time ($F_{(1, 59)} = 12.50$; $p < 0.001$), with no interaction of injury and time ($F_{(1, 59)} = 0.86$; $p = 0.356$). For hindlimb slips (Fig. 2b) there was a main effect of injury ($F_{(1, 59)} = 4.04$; $p = 0.049$), but not for time ($F_{(1, 59)} = 0.14$; $p = 0.709$), or an injury by time interaction ($F_{(1, 59)} = 1.82$; $p = 0.182$).

3.3. No differences in locomotion and anxiety-related behavior

Locomotor activity and anxiety-like behavior were assessed using open field at one day (n = 14/group) and 3.5 months (n = 17/group) following repeated sham/injury, with no differences found in total distance travelled (Supplementary Fig. 1a) and time spent in center zone (Supplementary Fig. 1b).

EPM was used to assess anxiety-like behavior at three days (n = 14/group) and 3.5 months (n = 17/group) after repeated sham/ACHI (Supplementary Fig. 2). For the duration of time spent in the open arms there was a main effect of time ($F_{(1, 58)} = 30.57$; $p < 0.0001$), however there was no main effect of injury ($F_{(1, 58)} = 0.00$; $p = 0.980$) and no injury by time interaction ($F_{(1, 58)} = 0.03$; $p = 0.853$).

3.4. Impaired spatial memory at acute, but not chronic, stages post-ACHI

The effect of repeated ACHI on spatial memory of the Y-maze was assessed at two days (n = 13/group) and 3.5 months (n = 16-17/group) post-final sham/ACHI (Fig. 3). Rats were excluded if they spent less than 10 s in each arm (n = 2) or escaped the maze (n = 1). For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury ($F_{(1, 55)} = 5.04$; $p = 0.029$), with no main effect of injury ($F_{(1, 55)} = 1.26$; $p = 0.267$) or time ($F_{(1, 55)} = 0.72$; $p = 0.399$). Post hoc analysis found a trend for repeatedly injured rats to spend less time in the novel when compared to the familiar arm at 2 days ($p = 0.056$), but not at 3.5 months post-final injury compared to sham rats ($p = 0.641$). Entries into the novel/familiar arms between sham and repeat ACHI rats was also assessed, revealing no main effect of injury ($F_{(1, 55)} = 0.60$; $p = 0.442$), time ($F_{(1, 55)} = 3.02$; $p = 0.088$), or injury by time interaction ($F_{(1, 55)} = 2.34$; $p = 0.132$).

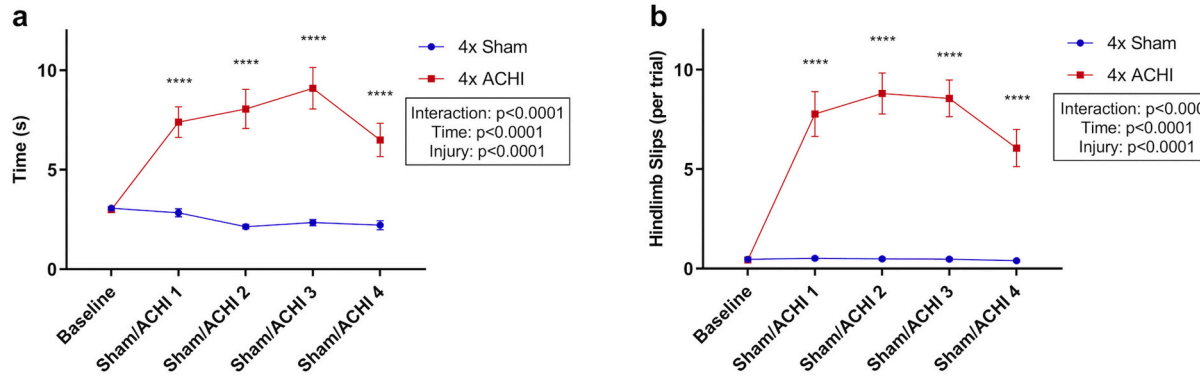

Fig. 1. Effect of repeat ACHI on sensorimotor function immediately after each injury. a) At 1 min after each ACHI, rats took significantly longer to traverse the beam compared to sham rats (ACHI 1–4, each $^{****}p < 0.0001$). b) Repeated ACHI rats also displayed more hind-limb slips compared to repeated sham rats (ACHI 1–4, each $^{****}p < 0.0001$). $n = 31$/group at each time-point. Data presented as mean ± SEM.

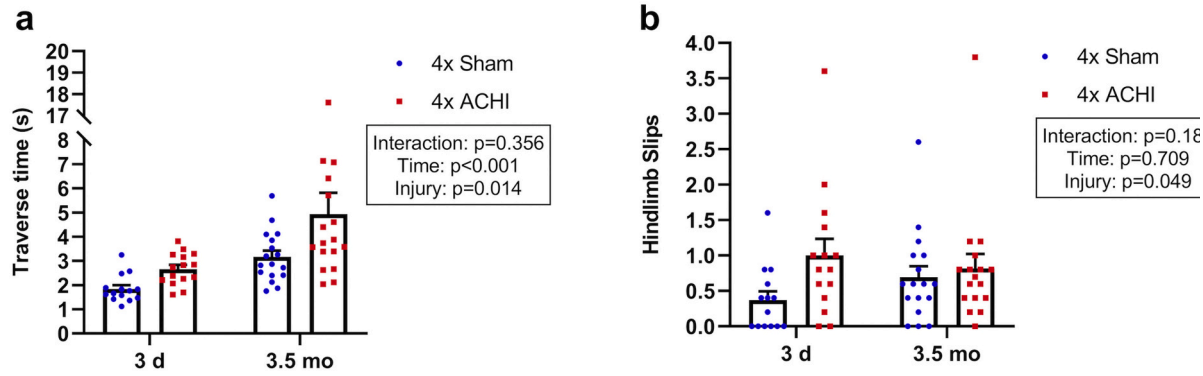

Fig. 2. Effect of repeat ACHI on sensorimotor function at 3 days and 3.5 months after final ACHI. a) A main effect was found in injury ($p = 0.018$) for beam traverse time at 3 days and 3.5 months after final-sham/ACHI b) For hindlimb slips per trial at 3 days and 3.5 months post-final ACHI, there was a main effect of injury ($p = 0.048$). n = 30-31/group at baseline, n = 14-15/group at 3 days, n = 16-17/group at 3.5 months. Data presented as mean + SEM.

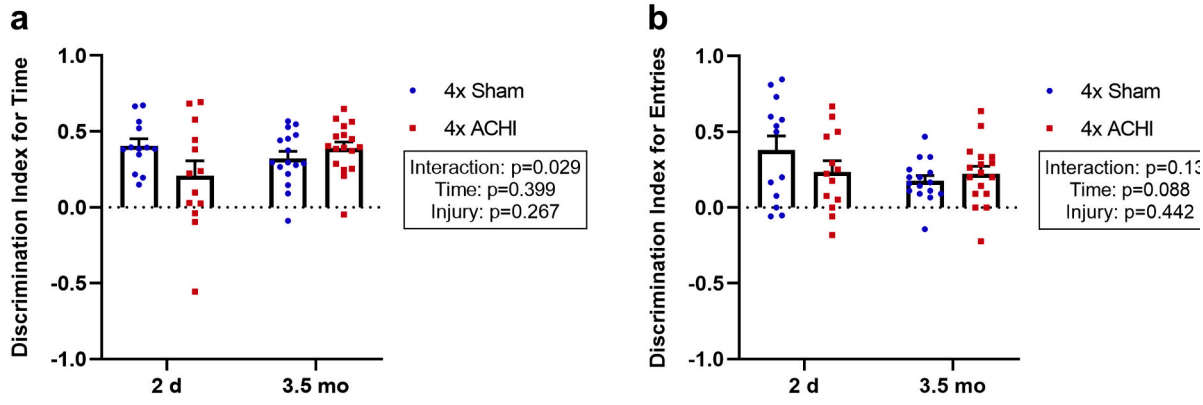

Fig. 3. Effect of repeat ACHI on spatial memory. a) For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury b) No significant differences were observed in novel arm entries between repeat ACHI and sham rats. n = 13-17/group. Data presented as mean + SEM.

Spatial memory at the chronic timepoint was also assessed using water maze (n = 17/group). In both acquisition and reversal testing, no differences were seen for latency to reach the platform, time in target quadrant, time in previous target quadrant (reversal), and direct and circle swims (Supplementary Fig. 3).

3.5. Acute but not chronic elevation in serum NfL levels

A Simoa HD-X Analyzer was used to quantify serum NfL levels at 7 days (n = 8) and 3.5 months (n = 8) after repeated sham or repeated ACHI (Fig. 4; Supplementary Fig. 4). Two-way ANOVA found a significant main effect of injury ($F_{(1, 27)} = 16.57$; $p < 0.001$), time ($F_{(1, 27)} = 6.40$; $p = 0.018$), and interaction of injury and time ($F_{(1, 27)} = 15.48$; $p < 0.001$) (Fig. 4a). Post hoc analysis revealed a significant difference in serum NfL between sham and ACHI rats at 7 days ($p < 0.0001$) but not 3.5 months ($p = 0.994$). A positive correlation (Pearson $r = 0.72$; $p = 0.044$) was found between 7-day serum NfL and the average number of beam slips immediately after each ACHI procedure (Fig. 4b; Supplementary Fig. 4a). A positive correlation was also revealed between 7-day serum NfL and beam traverse time at three days post-injury (Spearman r

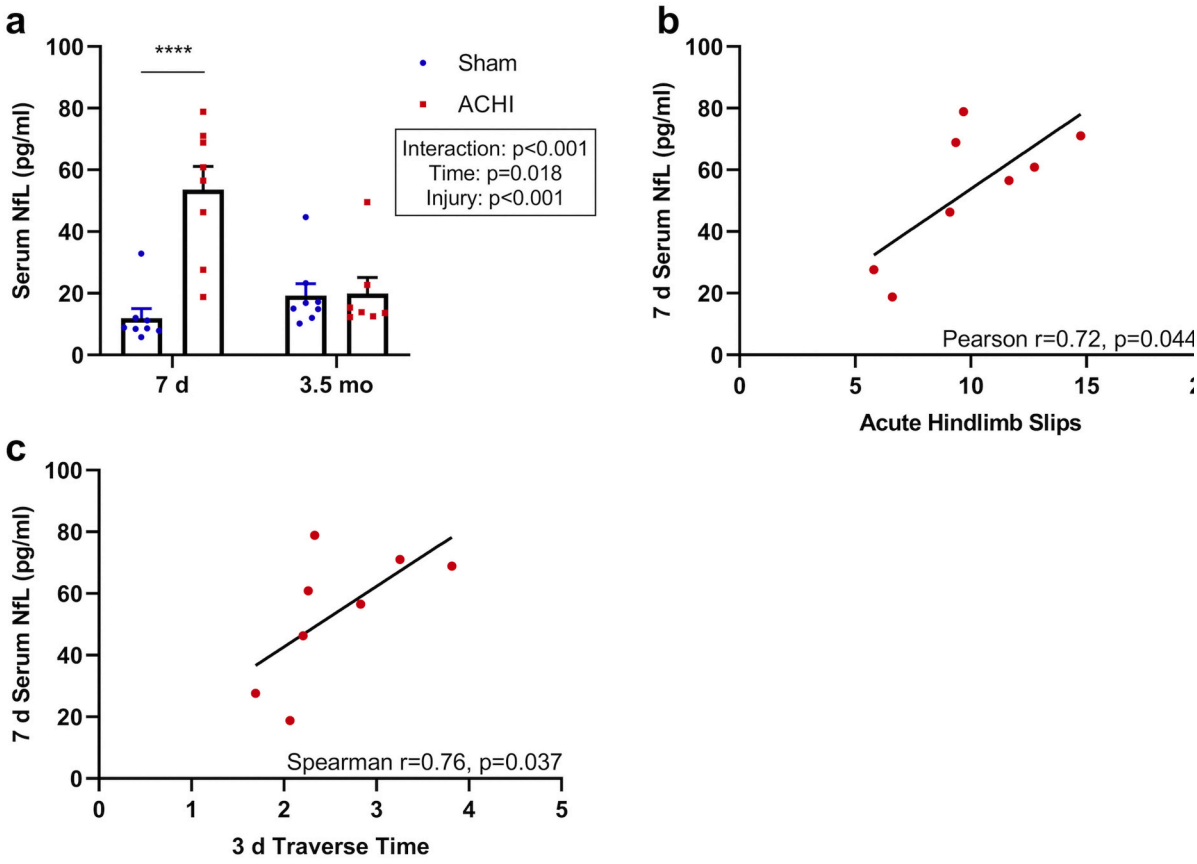

Fig. 4. Serum NfL levels after repeat ACHI. a) Serum NfL was significantly elevated at 7 days post-final ACHI (****$p < 0.0001$) but not at 3.5 months when compared to repeat sham rats. $n = 8$/group. Data presented as mean + SEM. b) A significant relationship between serum NfL levels at 7 days and average beam hindlimb slips immediately post ACHI at each timepoint (Pearson $r = 0.72$, $p = 0.044$). c) A significant correlation between serum NfL levels at 7 days and average beam traverse time at 3 days post ACHI (Spearman $r = 0.76$, $p = 0.037$). Linear regression line of best fit is plotted for visualisation purposes. n = 8/group.

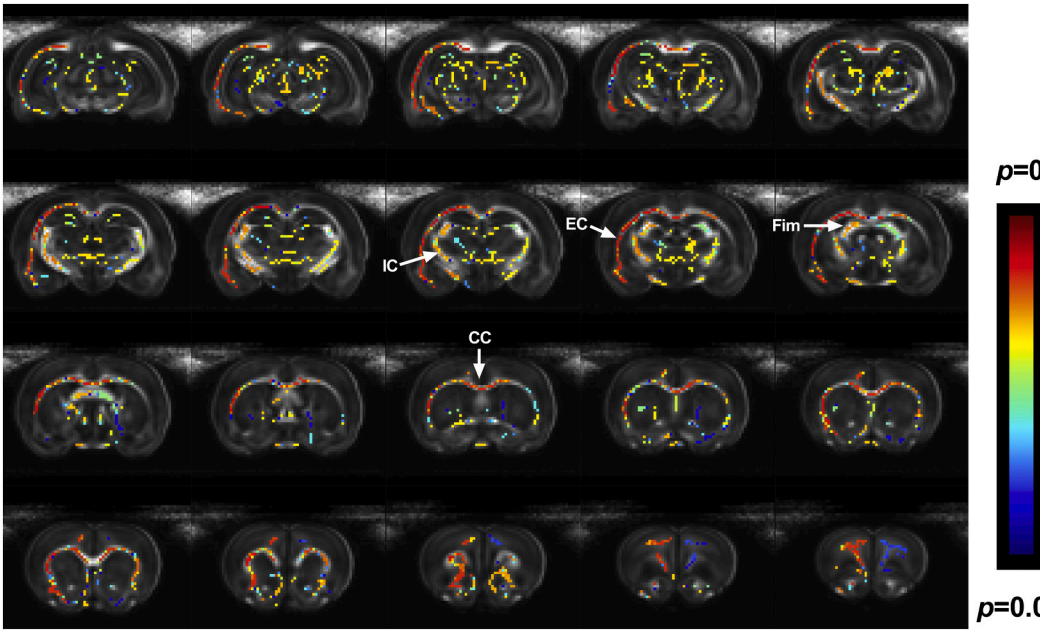

Fig. 5. Whole-brain TBSS revealed chronic FA changes in response to repeat ACHI. This figure depicts the FA study template (i.e. a representative FA image reconstructed from sham and mTBI rats in grayscale), with colored voxels representing significant ($p < 0.05$) group differences in FA between sham rats and mTBI rats at 3.5 months post-injury. Color bar indicates threshold-free cluster enhanced p-value. When compared to sham rats, repeated ACHI rats had reduced FA in voxels of various matter tracts. In particular, reduced FA appeared most evident in the corpus callosum, external capsule, internal capsule and fimbria of the hippocampus (indicated by white arrows). n = 8/group. CC = corpus callosum, EC = external capsule, IC = internal capsule, Fim = fimbria of the hippocampus.

= 0.76; $p = 0.037$) (Supplementary Fig. 4d). No other correlations were observed for beam performance and serum NfL at seven days or 3.5 months (Supplementary Fig. 4).

3.6. Widespread reductions in white matter FA

TBSS was used to assess FA of white matter tracts of ex vivo sham and repeat ACHI rat brains at 3.5 months (n = 8/group). Widespread, significant reductions in white matter FA were observed in both contralateral and ipsilateral hemispheres (Fig. 5). No volumetric differences were seen in the ipsilateral and contralateral cortex, corpus callosum and hippocampus between ex vivo scans of 3.5 month repeat sham and mTBI brains (results not shown).

3.7. Acute and chronic alterations in the hippocampal proteome

High-resolution, data-independent acquisition mass spectrometry was used to analyze the proteomic differences between fresh ipsilateral hippocampal tissue (n = 4-6/group) isolated from sham and repeated mTBI rats, and a total of 5053 proteins has been quantified across all samples. At seven days, 26 proteins were found to be significantly altered between sham and repeated mTBI rats (Supplementary Fig. 5a), and 72 proteins were significantly different in expression at 3.5 months (Supplementary Fig. 5b) when considering a false discovery threshold of 0.05. String database analysis of GO terms revealed 78 biological functions altered at seven days, and 182 altered at 3.5 months (data not shown). After reviewing GO terms, the Uniprot database (UniProt Consortium, 2018), and the literature, a selection of proteins deemed to have evidence for potential involvements in neurobiology and neuropathology were selected and presented in Table 1 (acute) and Table 2 (chronic).

4. Discussion

The neurobehavioral and pathophysiological alterations induced by repeated mTBI are still poorly understood. To provide new insights into the nature and progression of these changes, we implemented our clinically relevant rodent model of mTBI, along with detailed behavioral testing, blood and DTI biomarkers of axonal injury, and mass spectrometry of the hippocampal proteome. Our findings revealed that repeated ACHI was associated acutely with sensorimotor and subtle cognitive deficits, robust increases in serum NfL levels that were correlated with motor deficits, and increased levels of hippocampal proteins linked with changes in energy metabolism and glial activation. At the chronic stages there were subtle neurobehavioral deficits, widespread reductions in white matter integrity, and alterations in several hippocampal proteins including those associated with neurogenesis.

4.1. Behavioral deficits

Clinically, balance and gait are commonly assessed in acutely in patients suspected of suffering a mTBI (McCrory et al., 2017), with deficits usually resolving within days (Guskiewicz, 2001); however, there is some evidence that those with a history of mTBI may display prolonged subtle balance and gait deficits (Lynall et al., 2019; Sosnoff et al., 2011). Here, repeated mTBI resulted in significant sensorimotor deficits on the beam immediately after each impact, providing an indication of injury consistency. Analysis of beam performance at three days and 3.5 months revealed that repeated mTBI rats had sensorimotor impairments. Previously, we found that a single ACHI induced beam deficits that resolved within 24 h (Pham et al., 2019), thus indicating that repeat ACHI appears to have prolonged the duration of sensorimotor deficits. A main effect of time was found for time to traverse the beam, likely reflective of the increased age or size of the rats at the chronic time-point (Sun et al., 2019).

Impairments to memory are also common in clinical mTBI (McCrory et al., 2017), and repeated mTBI may cause cumulative and prolonged cognitive deficits (Broussard et al., 2018; Guskiewicz et al., 2005; Luo et al., 2014; Schatz et al., 2011). Y-maze testing revealed an injury by time interaction for relative time spent in the novel arm, providing some evidence of acutely impaired spatial memory in repeat mTBI rats. There were no deficits Y-maze and water maze test results at the chronic time-point. It is possible that tests conducted over multiple sessions (e.g. Morris water maze) may be required to detect more robust visuospatial memory deficits at acute stages and chronic time-points post-injury. Nonetheless, our findings indicate that in contrast to some other pre-clinical studies repeated mTBI did not result in overt and persisting spatial memory deficits (Shultz et al., 2019); however, differences in injury model, severity and schedule are likely to be contributing factors.

Table 1
Proteins of interest with altered ipsilateral hippocampal expression at 7-days following repeated mTBI compared to sham.

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
3-ketoacyl-CoA thiolase, peroxisomal	↑	Acetate CoA-transferase activity	Mitochondrial fatty acid beta-oxidation	(Chater-Diehl et al., 2016)
Annexin A2	↑↑	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	(Li et al., 2018; Zhao and Lu, 2007)
Calretinin	↑↑↑	Ca^{2+} binding	Ca^{2+} buffering. Regulation of presynaptic cytosolic Ca^{2+} concentration and long-term potentiation	(Buritica et al., 2009; Schmidt et al., 2013)
Collagen alpha-1(I) chain	↑↑↑	Fibrillar forming collagen	Component of glial scar	(Neo and Tang, 2017; Schwaller, 2014)
Dynein light chain 1, cytoplasmic	↓	Microtubule transport	Enables retrograde motility of vesicles and organelles along microtubules	(Gillardon et al., 1998)
ELAV-like protein 2	↑	RNA binding	Neurodevelopment and synaptic function.	(Berto et al., 2016; Zybura-Broda et al., 2018)
Electron transfer flavoprotein subunit beta	↑↑	Electron transfer activity	Mitochondrial fatty acid beta-oxidation and amino acid catabolism	(Shimazu et al., 2018)
60 kDa heat shock protein, mitochondrial	↑	Chaperonin involved in mitochondrial protein import	Mitochondrial protein import and macromolecular assembly. Promotes cytokine release and immune cell activation	(Dukay et al., 2019; Lehnardt et al., 2008)
Isocitrate dehydrogenase [NADP], mitochondrial	↑↑	Isocitrate dehydrogenase ($NADP^+$) activity	Mitochondrial enzyme that plays an essential role in intermediary metabolism and energy production	(Han et al., 2017; Kim et al., 2016)
Myosin-11	↑↑	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	(Rex et al., 2010)
Propionyl-CoA carboxylase beta chain, mitochondrial	↑	Propionyl-CoA carboxylase activity	Mitochondrial catabolism of odd chain fatty acids, branched-chain amino acids and other metabolites	(Tan et al., 2018)
Vimentin	↑↑	Class-III intermediate filaments of non-epithelial cells	Marker of reactive astrocytes. Promotes elongation of astrocytic processes and glial scar formation	(Ribotta et al., 2004; Wilhelmsson et al., 2004)

Table 2
Proteins of interest with altered ipsilateral hippocampal expression at 3.5 months following repeated mTBI relative to sham.

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
Alpha-actinin-4	↓	F-actin crosslinking protein	Important for cell motility and structural plasticity of neurons	(Kalinowska et al., 2015; Tentler et al., 2019)
Alpha-2-HS-glycoprotein	↓↓↓	Kinase inhibitor activity	Anti-inflammatory marker	(Shi et al., 2019; Wang et al., 2009)
Arachidonate 15-lipoxygenase	↓↓↓	Non-heme iron-containing dioxygenase	Generates anti-inflammatory molecules in hippocampal-prefrontal cortex. Depletion may compromise cognition	(Shalini et al., 2018)
Ankyrin-2	↑	Ion channel binding	Axonal domain organization and establishment. Essential for synaptic stability. Adaptor and scaffold for various neuronal ion channels	(Choi et al., 2019; Weber et al., 2019)
Annexin A2	↓↓↓	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	(Li et al., 2018; Zhao and Lu, 2007)
Sodium/potassium-transporting ATPase subunit beta-1	↑	Regulatory hydrolysis of ATP	Preservation of Na^{+}/K^{+} ATPases	(Wang et al., 2018; Wen et al., 2018)
Non-muscle caldesmon	↓↓↓	Actin binding protein	Enhances axon extension in hippocampal neurons	(Morita et al., 2012)
Contactin-1	↑	Nervous system development	Present at junctions of axons, myelin, and glial cells. Role in myelin sheath development	(Çolakoğlu et al., 2014)
Isocitrate dehydrogenase [NADP], mitochondrial	↓↓	Intermediary metabolism and energy production	Deficiency can cause oxidative stress and intensify mitochondrial dysfunction leading to neuronal death	(Han et al., 2017; Kim et al., 2016)
Integrin beta-1	↓↓↓	Collagen receptor	Itgb1 signalling promotes axon assembly. Removal causes axon formation deficits	(Huang et al., 2006; Lei et al., 2012)
Galectin-1	↓↓↓	Lectin, binding carbohydrates	Neuroprotection in ischemic brain injury	(Horie and Kadoya, 2002; Wang et al., 2015)
Lamin-B1	↓↓↓	DNA binding	Neuronal migration. Deficiency leads to abnormal neuronal development	(Mahajani et al., 2017)
Microtubule-associated protein 1B	↑	Microtubule binding	Present in axons and neurons. Essential for regulation of microtubule and synaptic interaction and stability	(Bodaleo et al., 2016; Gödel et al., 2015)
Myosin-11	↓↓	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	(Rex et al., 2010)
2-oxoglutarate dehydrogenase, mitochondrial	↓	Oxoglutarate dehydrogenase (NAD+) Activity	Impairment of Ogdh complex has key role in glutamate mediated neurotoxicity during TBI, leading to neuronal death	(Weidinger et al., 2017)
Tubulin beta-4B chain	↑↑	Structural component of cytoskeleton	Major structural constituent of microtubules. Important to neurodevelopment and plasticity	(Wu et al., 2009)

4.2. Axonal injury

4.2.1. TBSS

At 3.5 months, ex vivo MRI revealed no overt structural changes in all repeated mTBI rats, with volumes of the ipsilateral and contralateral cortex, corpus callosum and hippocampus all comparable to sham rats. Analyses of white matter integrity with TBSS did however reveal significant diffusion differences in repeated mTBI and sham rats, with reductions in FA observed in hemispheres ipsilateral and contralateral to the site of impact.

Reduced FA is commonly reported following mTBI in humans in both acute and chronic stages of injury (Dean et al., 2015; Rutgers et al., 2008; Wada et al., 2012; Wright et al., 2020). Although FA can also be increased by factors such as gliosis after TBI (Budde et al., 2011), reduced FA is widely thought to reflect white matter pathology such as axonal damage, demyelination, and reduced fibre density (Budde et al., 2011; Hutchinson et al., 2018). Although the extent of FA reductions appeared to be greater in the contralateral hemisphere, widespread changes in white matter structures were observed throughout the brain, with the corpus callosum, external capsule, internal capsule, and the fimbria of the hippocampus and corticofugal pathways particularly affected. Notably, these regions are commonly affected in clinical mTBI (Dean et al., 2015; Girgis et al., 2016; Messé et al., 2011; San Martín Molina et al., 2020), and we have previously found DTI changes in many of these regions in the acute stages after repeated ACHIs (Wortman et al., 2018). As such, damage to these regions may have been a key component of the cognitive deficits observed acutely, and the prolonged sensorimotor deficits. For example, the corpus callosum and corticofugal pathways are both thought to be critical to sensorimotor function, therefore damage to these regions may have contributed to the beam deficits in repeated mTBI rats (Li et al., 2015; Lindenberg et al., 2010). Nonetheless, further studies are required to understand the contribution of white matter changes to symptoms, as well as the temporal progression of these changes throughout acute, sub-acute stages after repeated mTBI.

4.2.2. Serum NfL

Quantification of serum NfL at seven days and 3.5 months after repeated mTBI allowed additional insights into the nature and progression of axonal damage. A cytoskeletal protein that is abundant in large-calibre myelinated axons, NfL is thought to be released into body fluids such as the cerebrospinal fluid (Shahim et al., 2016; Zetterberg et al., 2006) and blood (Shahim et al., 2018) following axonal injury or degeneration. Numerous studies have found serum NfL to be a dynamic biomarker of axonal pathology neurodegenerative diseases (Gaetani et al., 2019), with diffuse axonal injury hypothesised to be a key component of symptom outcomes following TBI (McKee and Daneshvar, 2015; Smith and Stewart, 2020).

In the present study, we found a robust increase of NfL concentrations in serum at seven days after repeat ACHI compared to repeat sham rats. This result is similar to findings in studies of boxers exposed to multiple head traumas, with increased serum NfL found at 7–10 days after competition (Shahim et al., 2017). Despite excellent rodent cross-reactivity of the Simoa NfL assay, relatively few pre-clinical studies have utilised this assay in the context of TBI (Cheng et al., 2019; Cheng et al., 2018). Our novel finding of robustly elevated NfL in serum at seven days after repeated mTBI indicates significant potential of this assay in pre-clinical research, and further indicates that the extent of axonal damage was likely substantial in the ACHI model. Moreover, increases in serum NfL correlated with acute sensorimotor deficits provides evidence of the potential of this biomarker as an indicator of mTBI severity.

A plethora of recent studies have found that serum NfL is highly sensitive to axonal damage in neurodegenerative diseases (Gaetani et al., 2019). Indeed, there is also emerging evidence that white matter damage may be a key contributing factor to some cases of PPCS (Khong et al., 2016; Miller et al., 2016). Given the associations with these conditions and repeated mTBI exposure, we hypothesised that serum NfL would remain elevated at 3.5 months post-injury; however, despite extensive white matter changes apparent at this stage, the resolution of this biomarker to sham levels is likely to indicate a lack of ongoing axonal damage. Future repeated mTBI studies are required to

understand if neurodegeneration is triggered at later points (e.g. 6–12 months post-injury) that may correspond to the typical age of onset of neurodegenerative conditions in humans (i.e. >60 years of age), and whether serum NfL levels can predict and monitor the rate and extent of potential neurodegeneration.

4.3. Hippocampal proteome

A variety of notable differences were found in the hippocampal proteome at seven days or 3.5 months post-injury. Although requiring further investigation, these findings shed new light into the short- and long-term effects of repeated mTBI.

4.3.1. Acute alterations

Unbiased mass spectroscopy revealed up-regulation of several mitochondrial proteins, particularly those associated with β-oxidation of fatty acids. Although the energy demands of the brain are primarily met by glucose (Mergenthaler et al., 2013), recent studies have indicated that capacity of the brain to oxidise fatty acids is greater than previously thought (White et al., 2020). As such, given that significant reductions in cerebral glucose metabolism have been reported in the days after single and repeated mTBI (Prins et al., 2013; Yoshino et al., 1991), it is possible that increased fatty acid catabolism represents a compensatory mechanism in order to meet the metabolic requirements of the injured brain. Furthermore, given the high lipid concentrations in the brain, increased β-oxidation may also represent a protective mechanism to minimize a potentially toxic build-up of fatty acids released by cellular damage. Future studies are required to test these hypotheses, and to understand whether β-oxidation of fatty acids may represent a novel target for neuroprotective therapies and biomarkers of injury neurobiology.

In addition to the proteins associated with adaptations to hippocampal energy metabolism, we also observed increases in proteins associated with gliosis and the formation of a glial scar, buffering of calcium, and maintenance of endothelial cell integrity. These findings provide further evidence that neuroinflammation, disrupted calcium homeostasis, and cerebrovascular damage, are all likely prominent in the acute and sub-acute stages after repeated mTBI.

4.3.2. Chronic alterations

We observed some increases in protein expression at 3.5 months after repeated mTBI that may be reflective of neurorestorative functions activated by repeated mTBI. For example, there is evidence that ankyrin 2 and contactin-1 are critical to formation and organization axons and synapses (Chatterjee et al., 2019; Koch et al., 2008; Weber et al., 2019). Contactin-1 also has a central role in oligodendrocyte myelin formation. Moreover, increases in microtubule-associated protein 1B and tubulin beta-4b, two key components of the axonal cytoskeleton, provide further evidence of potential activation of repair mechanisms after repeated mTBI.

In contrast, we also found a reduction in several proteins in animals subjected to repeated mTBIs that may be associated with impairments in neurodevelopment or neuroremodelling, or a reduced neurogenic capacity. For example, we observed reductions in caldesmon and myosin II, with the interaction of these two proteins previously shown to be critical to axonal extension in hippocampal neurons (Morita et al., 2012). In addition, integrin beta-1 is a cell surface receptor thought to have diverse and important roles in the central nervous system (Panov et al., 2014), including outgrowth of hippocampal progenitor cells (Harper et al., 2010) and axon assembly during development (Lei et al., 2012), with multiple studies demonstrating that deficiency of integrin beta-1 results in impaired long-term potentiation (Huang et al., 2006), synaptic plasticity and working memory (Chan et al., 2006). Although further research is required to confirm and understand the significance of these decreases in protein expression, when considering the significant literature on their neurobiological importance, we speculate that these proteomic changes may contribute to chronic neuropathology and behavioral impairments after repeated mTBI.

Although this study has provided some important new insights into the potential effects of repeated mTBIs, there are some limitations to the study that should be acknowledged. Firstly, it is important to note that given this is a single sex study, there may be limitations in generalizing the current findings to females. Second, although we chose not to run acute behavioral analysis on the chronic recovery rats to avoid the potential confound of repeated task exposure, serial testing may have provided greater insights into the temporal progression of behavioral phenotypes after repeated mTBI. In addition, TBSS analysis was chosen to enable unbiased assessment of white matter throughout the brain structures; however, future studies could apply ROI analysis to examine changes in defined brain structures. Using this approach, one could also examine how DTI changes in specific brain structures relate to other functional (e.g. sensorimotor deficits) and molecular (e.g. NfL) changes. However, serum NfL levels appear to normalize by the chronic stages of injury, and as such, we hypothesize that correlation with FA values appears unlikely. Although we observed robust changes in white matter with TBSS, further studies are required to validate these changes with complementary techniques such as histology. Finally, proteomic analysis in this study was limited to the ipsilateral hippocampus and analysis with mass spectrometry, therefore further studies are required to validate these changes and investigate potential alterations in other brain regions.

5. Conclusions

With increasing concern that exposure to repeated mTBIs can lead to lingering or progressive neurological impairments, it is imperative that pre-clinical research is conducted to better understand these associations and their pathophysiological underpinnings. Using a clinically relevant rat model of repeated mTBI, our acute recovery studies revealed that while still in the symptomatic period, rats had changes in several hippocampal proteins suggestive of adaptations in energy metabolism and increased glial reactivity, as well as a profound increases in serum axonal injury biomarker NfL that correlated with sensorimotor deficits. At a chronic stage of injury, despite displaying minimal neurobehavioral deficits and a return of NfL to sham levels, we found significant disruptions to the hippocampal proteome, and widespread reductions in white matter integrity. Given the significant promise of serum NfL as an early indicator of neurodegenerative disease onset, we speculate that resolution of this biomarker indicates that changes in white matter were not progressive at 3.5 months post-injury, with future studies required to determine if progressive neurodegenerative changes are apparent at a more chronic time-points.

Author contributions

LP conducted all behavioral experiments, performed tissue collection and preparation, generated the data and wrote the draft manuscript. SRS and SJM conceptualized and supervised the project. WTO assisted with individual experiments. DKW generated and analyzed MRI data and wrote the text of the final manuscript. RBS, CH and ADS performed mass spectrometry, analyzed the data and wrote the text of the final manuscript. PMC, MS and JB critically analyzed data. CGS supervised the project. LP, SRS, SJM, DKW, WTO, MS, PMC, JB, RBS, CH, ADS, CGS, RDB, TJO and RM reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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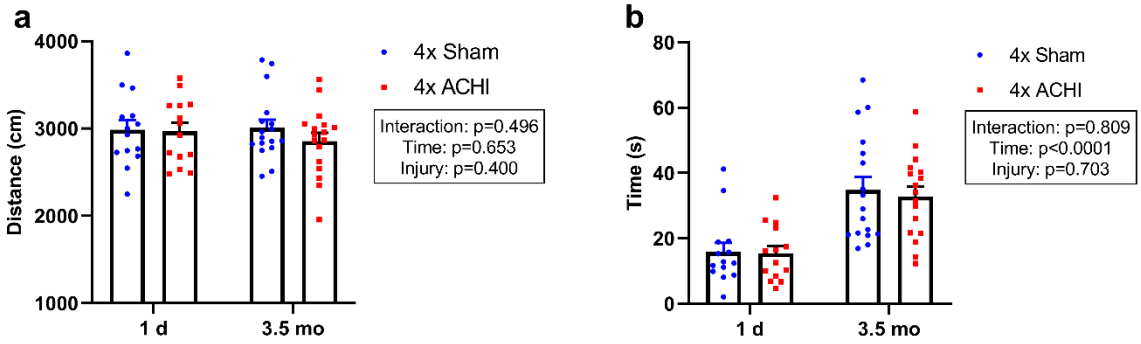
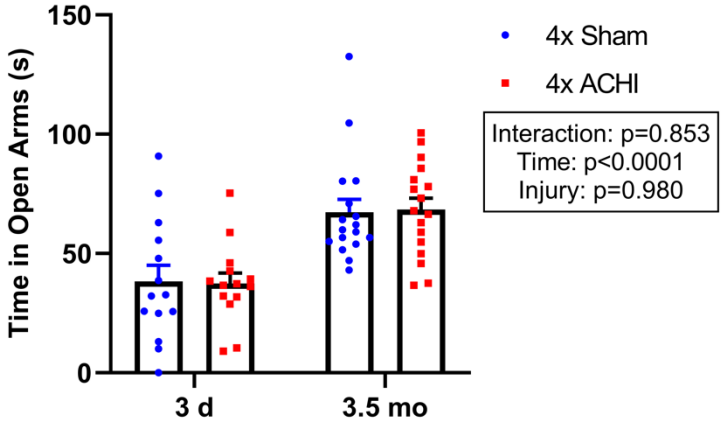
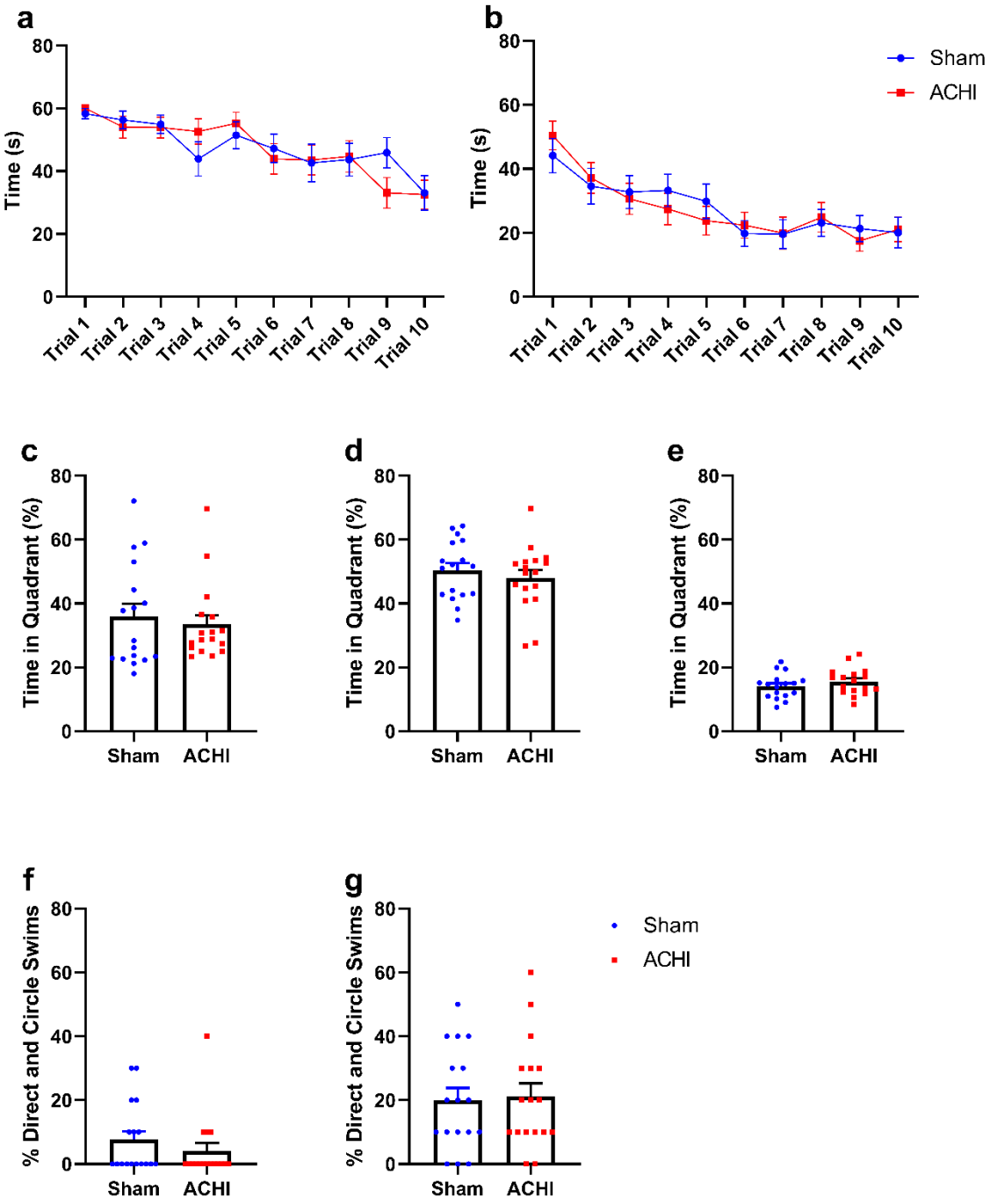
Supplementary Data



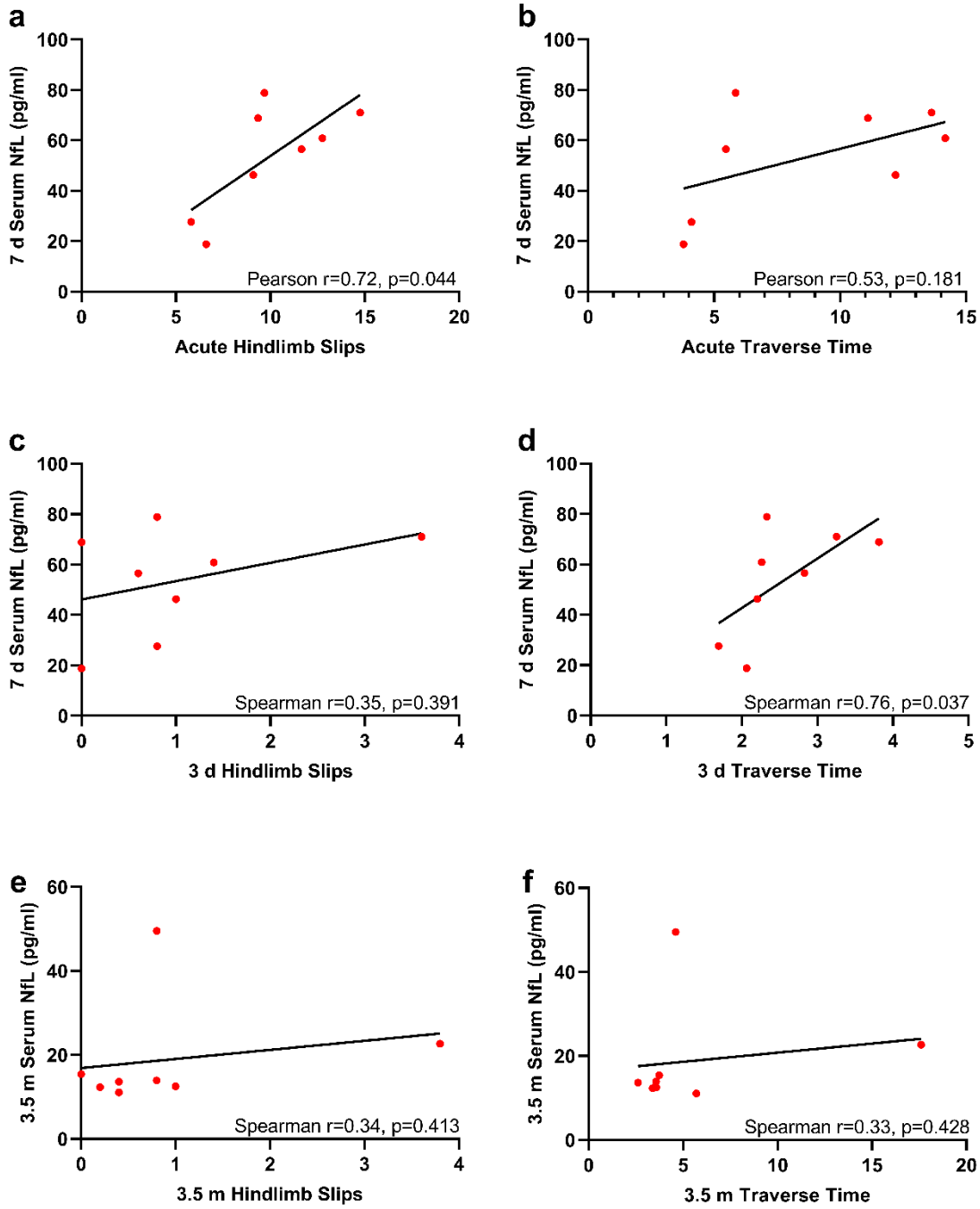
Supplementary Fig. 1. Locomotor and anxiety-like behaviour after repeat ACHI. Open field revealed no differences between repeat sham ACHI rats in **a)** distance travelled and **b)** time spent in centre at both 1 day and 3.5 months after final repeat sham/ACHI. n=14-17/group at each time-point. Data presented as mean + SEM.



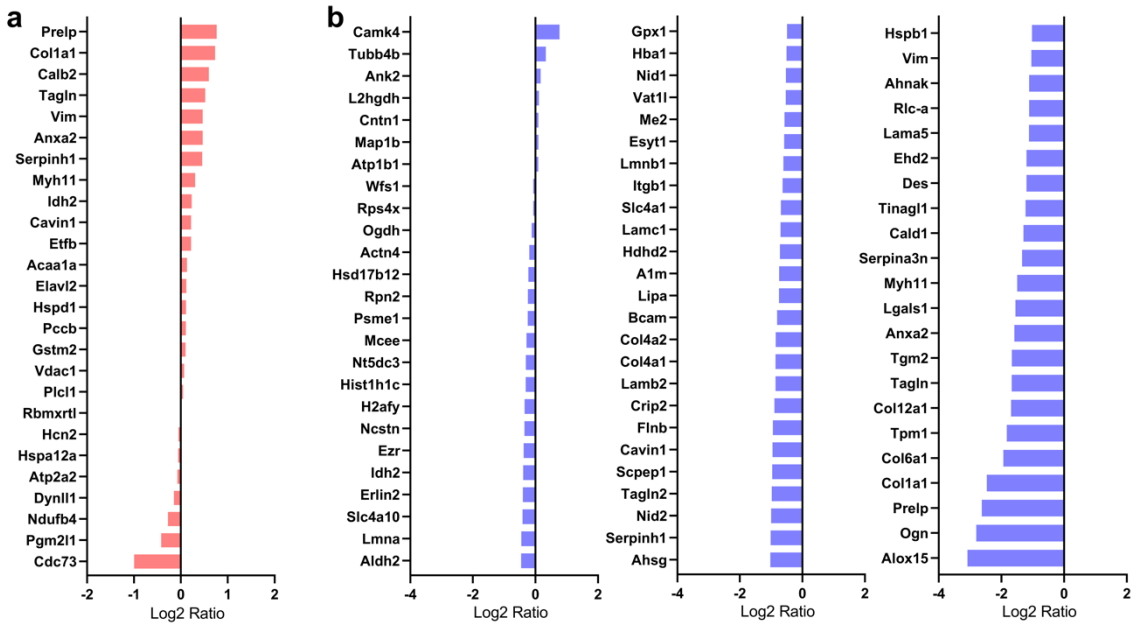
Supplementary Fig. 2. Effect of repeat ACHI on anxiety-like behaviour. No differences between repeat sham ACHI rats were found in time spent in open arms at both 3 days and 3.5 months after final repeat sham/ACHI in the elevated plus maze. n=14-17/group at each time-point. Data presented as mean + SEM.



Supplementary Fig. 3. Chronic effects of repeat ACHI on spatial working memory. No differences were observed at 3.5 months post-final sham/ACHI in **a)** latency to platform at acquisition or **b)** reversal between repeat sham and ACHI rats. n=17/group. Data presented as mean ± SEM. No differences were seen in **c)** time spent in target quadrant at acquisition, **d)** reversal and **e)** percentage of time spent in old target quadrant between repeat sham and ACHI rats at 3.5 months post-final sham/ACHI. No differences between repeat sham/ACHI rats were seen in **f)** direct or circle swims at acquisition and **g)** reversal. n=17/group. Data presented as mean + SEM.



Supplementary Fig. 4. Effect of repeat ACHI on serum NfL levels. **a) Serum** NfL levels at 7 days correlated with average beam hindlimb slips immediately post-ACHI at each timepoint (Pearson r=0.72; p=0.044), but did not correlated with average traverse time to immediately post-ACHI (**b**; Pearson r=0.53; p=0.181), 3-day hindlimb slips (**c**; Spearman r=0.35; *p*=0.391) or traverse time (**d**; Spearman r=0.76; *p*=0.037). Serum NfL levels at 3.5 months did correlate with beam slips (**e**; Spearman r=0.34; *p*=0.413) or traverse time (**f**; Spearman r=0.33; *p*=0.428) at this chronic time-point. n=8 at each timepoint.



Supplementary Fig. 5. Alterations in the hippocampal proteome. **a)** Proteins of the ipsilateral hippocampus of **a)** 7 day and **b)** 3.5 months post-repeat sham/ACHI with Q-value less than 0.05 revealed by mass spectroscopy. n=4-6/group. Data presented in log2 ratios.