Acute Neurobehavioural Changes Following Repeat Mild Traumatic Brain Injury

by

Ryan C. Wortman
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Supervisory Committee

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Dr. Brian R. Christie (Division of Medical Sciences)
Supervisor

Dr. Leigh Anne Swayne (Division of Medical Sciences)
Departmental Member

Dr. Robert L. Chow (Department of Biology)
Outside Member

Abstract

Supervisory Committee
Dr. Brian R. Christie (Division of Medical Sciences)
Supervisor
Dr. Leigh Anne Swayne (Division of Medical Sciences)
Departmental Member
Dr. Robert L. Chow (Department of Biology)
Outside Member

There is increasing evidence that repeat mild traumatic brain injury (rmTBI) may result in cumulative and long-term symptoms, more pronounced behavioural deficits, and neurodegeneration. Children have a greater susceptibility to head injury and represent a significant at risk population for rmTBI, especially those that participate in contact sports. Despite this, there is a paucity of data on rmTBI pathophysiology in the juvenile brain. The current study utilizes a novel awake closed head injury (ACHI) model to deliver repeat injuries to fully conscious juvenile rats. The ACHI model avoids the potential confounds of anaesthesia, and facilitates the assessment of neurological function immediately after each impact. Results indicate that the ACHI model produces acute neurological deficits after each impact, and that repeat injury worsens outcomes. Behavioural testing identified transient anxiety-like behaviour and motor impairment in response to rmTBI. The functional impairments and affective behaviour were in the absence of tau protein pathology. This study represents the first investigation of the consequences of rmTBI on the juvenile brain using an awake model of brain injury.

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ACHI – awake closed head injury
AD – Alzheimer's disease
AMPA – D-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
BAD – Bcl-2-associated death promoter
BBB – blood-brain barrier
BCA – bicinchoninic acid
BSA – bovine serum albumin
APS – ammonium persulphate
ATP – adenosine triphosphate
Ca^{2+} – calcium
CA – *cornu ammonis*
cAMP – cyclic adenosine monophosphate
CB – cerebellum

CCI – controlled cortical impact
CHI – closed head injury
CDK5 – cyclin-dependent kinase 5
CREB – cAMP response element binding protein
CSF – cerebrospinal fluid
CT – computed tomography
CTE – chronic traumatic encephalopathy
CTX – cortex
DAI – diffuse axonal injury
DG – dentate gyrus
DTI – diffusion tensor imaging
DTT – Dithiothreitol
ECL – enhanced chemiluminescence
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
EPM – elevated-plus maze
ERK1/2 – extracellular signal-related kinase 1/2
FPI – fluid percussion injury
FST – forced swim test
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GCS – Glasgow coma scale
GSK-3 – glycogen synthase kinase-3

GSK-3α – glycogen synthase kinase-3 α isoform
GSK-3β – glycogen synthase kinase-3 β isoform
HPC – hippocampus
HRP – horseradish peroxidase
ICP – intracranial pressure
IHC – immunohistochemistry
JNK – c-Jun N-terminal kinase
K^+ – potassium
LTP – long-term potentiation
MABP – mean arterial blood pressure

MAP – microtubule-associated protein
MAPK – mitogen-activated protein kinase
MRI – magnetic resonance imaging
MT – microtubule
mTBI – mild traumatic brain injury
Na^+ – sodium
NMDA – N-methyl-D-aspartate
NSS – neurological severity score

NF-Kb – nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT – nuclear factors of activated T-cells
NFT – neurofibrillary tangles
OF – open-field
PBS – Phosphate-buffered saline
PFC – pre-frontal cortex
PHF – paired helical filaments
PID – post injury day
PI3K – phosphoinositide 3-kinase
PKA – protein kinase A
PKB – protein kinase B
PKC – protein kinase C
PND – postnatal day
PP1 – protein phosphatase 1
PP2A – protein phosphatase 2A
PP2B – protein phosphatase 2B
PTSD – post-traumatic stress disorder
PVDF – Polyvinylidene Fluoride
rmTBI – repeat mild traumatic brain injury
RPPM – Reverse phase protein microarray
RR – Rota-Rod

SDS – sodium dodecyl sulfate
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser202 – Serine-202 (of tau protein)
Ser473 – (of Akt)
Ser9 – Serine-9 (of GSK-3β)
TBI – traumatic brain injury
TBS – Tris Buffered-Saline
TEMED – tetramethylethylenediamine
WB – Western blot

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Dedication

For Rowen, who was too young, and for all those affected by concussion, brain injury, and CTE. I hope that what we do here in the laboratory will one day make a difference.

&

To my parents. Thank you for your unwavering support in all of my academic endeavours. None of this would be possible without you and your help, guidance, and love.

1. Introduction

1.1 Traumatic Brain Injury

Damage to the brain resulting from an external mechanical force, such as an impact, compression, blast wave, rapid change in acceleration or penetration by a projectile, is defined as a traumatic brain injury (TBI). This form of trauma is a major worldwide health and socioeconomic concern, which represents the foremost cause of mortality and disability for individuals 45 years of age and under (Ghajar, 2000; Cole, 2004). The leading causes of TBI are falls, traffic accidents, violence, and sports-related injury (Bruns and Hauser, 2003). Each year, a global incidence of 10 million hospitalizations and/or deaths are the direct result of TBI with 57 million currently living people estimated to have experienced such an injury (Langlois et al., 2006). Of those hospitalized, nearly half (43%) have a TBI-linked disability one year after the injury (Selassie et al., 2008). TBIs may produce a variety of transient or long-term symptoms that affect cognitive and/or motor function, as well as sensory abilities and emotions (Thurman et al., 2007).

1.2 TBI pathophysiology

TBI is a complex, chronic disease process that is not limited to a single pathophysiological event (Masel and DeWitt, 2010), and involves structural damage and functional deficits resulting from both primary and secondary injury mechanisms (Davis, 2000). The primary injury is the direct result of the external force, and typically involves mechanical tissue deformation, blood vessel damage, and axonal shearing and stretching (Povlishock and Christman, 1995; Gaetz, 2004). Diffuse axonal injury (DAI) is a hallmark of TBI, and likely underlies the cognitive deficits and disability post-injury (Smith et al., 2003). The secondary injury is the result of the primary injury and evolves over days to months. Biochemical changes involved in the secondary injury include glutamate excitotoxicity, altered calcium (Ca^{2+})

homeostasis, inflammation, apoptosis, mitochondrial dysfunction and DAI, as well as increased free radical production and lipid peroxidation (reviewed by Povlishock and Christman, 1995). The secondary injury is the result of metabolic, molecular, and cellular cascades that eventually leads to neuronal death, tissue damage, and brain atrophy (Marklund et al., 2006; Bramlett and Dietrich, 2007). In short, TBI causes a constellation of biochemical changes in the brain that result in an energy crisis in the damaged cells leading to impaired neurotransmission, and cytoskeletal changes, which leads to chronic dysfunction of axonal transport and ultimately cell death (Giza and Hovda, 2001, 2014).

1.3 Classification and Diagnosis

TBI classification may vary, but is most commonly classified on the basis of clinical severity in the emergency room (Stein, 1996). The Glasgow Coma Scale (GCS) represents a universally accepted tool for clinical assessment of injury that is based on the level of consciousness of the patient. The severity of TBI is determined by a sum score of eye, motor, and verbal responsiveness, ranging from 3-15. Injuries are classified as severe (GCS 3-8), moderate (GCS 9-13), or mild (GCS 14-15). The likelihood of hospitalization and length of stay is a factor of injury severity (Teasdale and Jennett, 1974).

TBI is a heterogeneous disorder that can be caused by a variety of external factors. The nature, direction, duration, and location of the force of impact influences the outcome and severity of damage (Maas et al., 2008). Additional factors such as age, sex, health, medication, drug use, and genetics also influence injury symptoms and pathophysiology (Margulies and Hicks, 2009). As a result, both the diagnosis and prognosis of TBI are difficult and largely restricted to subjective assessments, especially with a mild head injury where patients may have no obvious clinical symptoms. Self-assessment of symptoms or with clinical tools such as the

GCS can be obscured by motive, and require the patient to be conscious (Balestreri et al., 2004). Level of consciousness may also be altered by confounds such as sedation, paralysis, or intoxication (Balestreri et al., 2004). The utilization of neuroimaging represents a more standardized approach to clinical diagnosis that are not influenced by such confounds. Magnetic resonance imaging (MRI) (Firsching et al., 2001) and computerized tomography (CT) (Uchino et al., 2001; Zhu et al., 2009) are two imaging methods that aid in diagnosing TBI. MRI and CT are less subjective, but are costly, time-consuming, and are primarily used only in severe cases due to limited sensitivity (Sandler et al., 2010).

1.4 Mild TBI

Mild TBI (mTBI) is synonymous with concussion, and the terms are used interchangeably. mTBI is defined, according to the 4th International Conference on Concussion in Sport (Zurich, 2012), as a complex pathophysiological process affecting the brain induced by biomechanical forces. The injury may be caused by either a direct or indirect blow to the head. Symptoms are typically rapid onset, short-lived, and resolve spontaneously. The acute clinical symptoms are the result of a functional disturbance rather than a structural injury, as no abnormalities are seen with neuroimaging. Lastly, mTBI may or may not involve loss of consciousness (McCrory et al., 2013).

Early clinical symptoms of mTBI may include, but are not limited to, headaches, dizziness, confusion, nausea, and vomiting. Later symptoms, on the time scale of days to weeks, include persistent headaches, balance problems, attention deficits, sleep disturbances, learning and memory problems, and irritability (Anon, 1997; Prins et al., 2010; Caine et al., 2014). The most prominent feature of mTBI is cognitive impairment, which manifests as learning and memory deficits (Baddeley et al., 1987; Dikmen et al., 1987; Ylvisaker and Feeney, 1998).

Amnesia of events directly before or after the trauma occur in 30% of mTBI patients (Guskiewicz et al., 2001). Approximately 40-80% of individuals that sustain a mTBI will present some variation of clinical symptoms, and may additionally suffer from fatigue, anxiety, affective lability, and cognitive impairment (Hall et al., 2005). In most cases, individuals recover from physical, cognitive, and psychological symptoms within 7-10 days, and no longer than three months (McCrory et al., 2013). In a subset of individuals (10-20%), mTBI symptoms can persist for over a year (McCrea et al., 2003; Hall et al., 2005). The neurobehavioural sequelae associated with mTBI is discussed in greater detail in Section 1.9.

1.4.1 Epidemiology

mTBI, considered a 'silent epidemic' of modern times (Tellier et al., 1999), accounts for upwards of 80% off all head injuries (Bernstein, 1999; Anon, 2009) and is 10-fold more prevalent than other TBIs (Bazarian et al., 2005). The incidence of mTBI is currently estimated to be 100 - 300 people per 100,000 based on those that seek medical attention (Cassidy et al., 2004). In reality, many people who sustain a mTBI will not seek medical care (Setnik and Bazarian, 2007). As such, the global incidence is likely greater than 600 per 100,000 people, or roughly 42 million globally that sustain a mTBI annually (Cassidy et al., 2004). Certain sub-populations such as hockey, rugby, football, boxing, and soccer athletes, as well as military personnel are at particularly high risk for sustaining a mTBI (Cassidy et al., 2004). Young males between the age of 15-24 represent the most at risk population, and sustain mTBI 2-3 times more frequently than females (Weight, 1998; Bernstein, 1999).

1.4.2 Neurobiology

In immediate response to the biomechanical forces of injury, axonal stretching, disruption of neuronal membranes, and the opening of voltage-dependent potassium (K^+) channels leads to increased extracellular K^+ (Takahashi et al., 1981; Ballanyi et al., 1987; Katayama et al., 1990). Additionally, nonspecific depolarizations cause indiscriminate release of the excitatory amino acid glutamate, which further contributes to the K^+ dysregulation via activation of kainate, N-methyl-D-aspartate (NMDA), and D-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (**Figure 1, part 1, 2**). Excess extracellular K^+ triggers neuronal depolarization causing additional release of glutamate, activation of kainate, NMDA, and AMPA receptors, and subsequent K^+ efflux (**Figure 1, part 3**). This significant state of excitation is followed by diffuse neuronal depression (Prince et al., 1973; Sugaya et al., 1975).

In order to restore ionic homeostasis, sodium-potassium (Na^+/K^+) pump activity is increased (Mayevsky and Chance, 1974) and requires significant amounts of adenosine triphosphate (ATP) are required to power the pumps. This increased energy demand triggers an increase in glucose usage (Shah and West, 1983), consequent hyperglycolysis, and results in increased lactate production and accumulation (**Figure 1, part 4, 5, 6**) (Nilsson and Pontén, 1977; Yang et al., 1985). Elevated levels of lactate further complicate neuronal dysfunction by inducing altered blood-brain barrier (BBB) permeability, acidosis, and cerebral edema (Kalimo et al., 1981a, 1981b; Gardiner et al., 1982).

Activated NMDA receptors flux Ca^{2+} into the cell. Ca^{2+} is a universal second messenger implicated in a multitude of signalling pathways. However, increased Ca^{2+} following trauma may cause cell death via over activation of calpains (Roberts-Lewis and Siman, 1993; Kampfl et al., 1997), phospholipases (Farooqui and Horrocks, 1991), or protein kinases (Verity, 1992). Alterations in Ca^{2+} signaling has also been linked to neurofilament degradation (Iwasaki et al.,

1987), free radical overproduction (Siesjö, 1992), and activation of pro-apoptotic genes (Morgan and Curran, 1986). In addition, excess intracellular Ca^{2+} is sequestered in the mitochondria, which results in mitochondrial dysfunction and consequent impairment in oxidative metabolism after brain injury (Verweij et al., 1997; Xiong et al., 1997). Impaired oxidative metabolism may result in decreased ATP production, thereby exacerbating the energy crisis and further contributing to hyperglycolysis (**Figure 1, part 5, 7, 8, 9**).

DAI is a direct result of the acceleration/deceleration forces of head trauma that causes shearing and tearing of axons (Adams et al., 1989; Alexander, 1995; Meythaler et al., 2001). DAI is the primary neuropathology of TBI in general, and may result in membrane disruption and even depolarization (Julian and Goldman, 1962). Disruption of the axolemma causes increased permeability (Pettus et al., 1994; Povlishock and Pettus, 1996), Ca^{2+} influx, and mitochondrial swelling (Mata et al., 1986; Maxwell et al., 1995) (**Figure 1, part A**). The increased axonal Ca^{2+} levels have been shown to trigger microtubule breakdown post-injury (Pettus and Povlishock, 1996; Maxwell and Graham, 1997). Neurofilament compaction and potential collapse has also been identified (Sternberger and Sternberger, 1983; Nakamura et al., 1990; Nixon, 1993). Collectively, the cytoskeletal abnormalities cause accumulation of axonal transport products that results in axonal swellings and secondary axotomy (**Figure 1, part B, C, D**).

DAI has previously been identified in patients with TBI (Oppenheimer, 1968), although the severity of DAI has been found to be proportional to the deceleration force of impact (Elson and Ward, 1994). Until recently, DAI pathology following mTBI was not detectable using conventional neuroimaging techniques. However, advances with diffusion tensor imaging (DTI) have now identified DAI in mTBI patients (Bazarian et al., 2007; Miles et al., 2008; Mayer et al.,

2010) and even in response to sub-concussive blows (Bazarian et al., 2012). Further DTI studies have shown that the extent of DAI following mTBI is related to the development of cognitive impairment (Lipton et al., 2008; Niogi et al., 2008; Wilde et al., 2008).

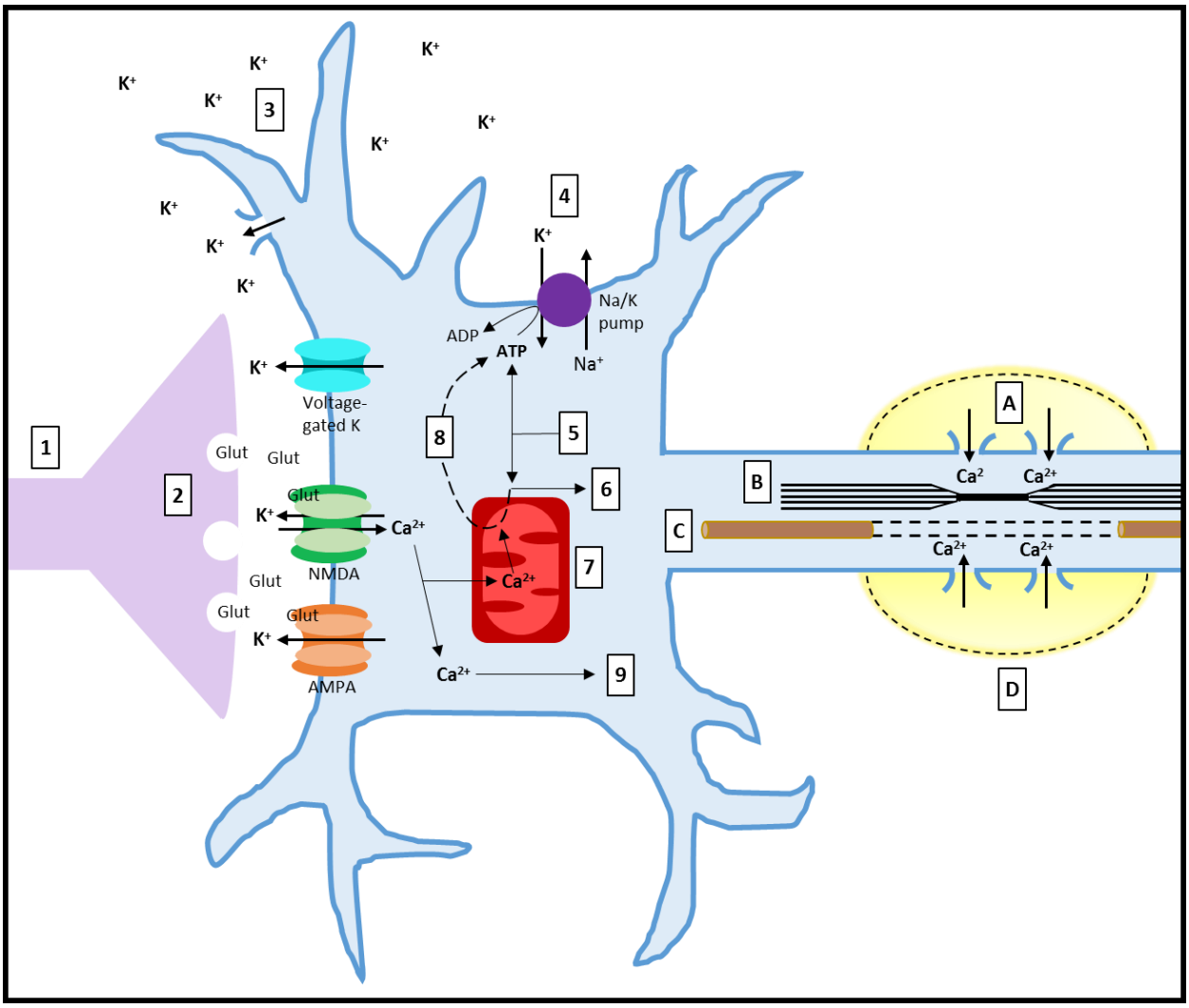

Figure 1. Neuropathophysiology of mild traumatic brain injury. (1) Nonspecific depolarizations and dysregulated firing of action potentials. (2) Indiscriminate release of the excitatory neurotransmitter glutamate. (3) Large potassium efflux. (4) Upregulation of sodium-potassium pump to restore ionic homeostasis. (5) Hyperglycosis to sustain ATP demand. (6) Lactate accumulation as a by-product of hyperglycolysis. (7) Calcium influx; sequestering of calcium in the mitochondria that results in impaired oxidative phosphorylation. (8) Decreased ATP production. (9) Activation of calpain signaling leading to initiation of apoptosis. A, Calcium influx in response to disruption of the axolemma. B, Neurofilament compaction. C, Microtubule disassembly. D, Axonal swelling and secondary axotomy. K^+, potassium; Glut, glutamate; NMDA, N-methyl-D-aspartate; AMPA, d-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; Ca^{2+}, calcium; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Na^+, sodium. Image adapted from Giza and Hovda, 2002.

1.5 Repeat mTBI

In response to mTBI, a cascade of cerebral pathophysiology is initiated (**Figure 1**), which is thought to be responsible for the transient neurological and cognitive impairments associated with injury (Barkhoudarian et al., 2011). These transient functional changes may be due to disrupted ionic homeostasis, glucose excitotoxcity, and/or altered cerebral blood flow and metabolism (Hovda et al., 1991; Yoshino et al., 1991a). Such dysregulation, either independently or collectively, may put the brain in an enhanced state of vulnerability whereby a second injury could exacerbate the damage (Jenkins et al., 1989; Hovda et al., 1991; Yoshino et al., 1991a; Gennarelli, 1993). It is now known that a prior mTBI event puts the patient at greater risk of sustaining additional head injuries, or repeat mTBI (rmTBI) (Zemper, 2003; Barkhoudarian et al., 2011; MacGregor et al., 2011; Dams-O'Connor et al., 2013; Tremblay et al., 2013). Additionally, repeat head injuries may result in cumulative damage, and recovery from a concussion is prolonged if a prior concussion has occurred (Salcido and Costich, 1992; Elson and Ward, 1994; Kelly and Rosenberg, 1998). Sub-populations that play contact sports such as ice hockey, boxing, soccer, and football are particularly susceptible (Tysvaer et al., 1989; Cantu, 1996; Matser et al., 1999), as well as victims of domestic violence (Roberts et al., 1990) or child abuse (Duhaime et al., 1998; Lancon et al., 1998).

1.5.1 Clinical rmTBI

Clinical evidence of patients with history of multiple concussions have identified increased learning and memory disabilities (Bijur et al., 1996; Matser et al., 1998; Collins et al., 1999; Wall et al., 2006), slowed balance recovery (Slobounov et al., 2007), decreased information processing rates (Gronwall and Wrightson, 1975), impaired visuospatial perception (Matser et al., 1998), difficulty in concentration, and increased incidence of headaches (Gaetz et al., 2000). Symptoms of mTBI usually resolve spontaneously within 2-3 weeks (Lovell et al.,

2003; McCrea et al., 2003), whereas repeat injuries may cause symptoms to persist for extended periods of time (Pellman et al., 2003; Arciniegas et al., 2005; Halstead and Walter, 2010). In fact, increasing evidence suggests a link between recurrent concussions in young adulthood and early onset cognitive and behavioural impairment (Guskiewicz et al., 2005). Moreover, rmTBI may actually increase the risk of developing dementia (Guskiewicz et al., 2005) and neurodegenerative diseases (McKee et al., 2009; Masel and DeWitt, 2010).

Indeed, athletes represent a significant at-risk population for sustaining rmTBI based on the nature of contact sports. Consequently, athletes and sports-related injuries make up a large proportion of the clinical rmTBI research population. Athletes that have suffered previous concussions are significantly more likely to sustain a second concussion (Cantu, 1996; Chorley, 1998), especially within 7 to 10 days of the first injury (Guskiewicz et al., 2003). It is important to note, however, that repeat head injury is not limited to professional leagues, but also impacts juvenile and amateur athletes at all levels of play (Mendez, 1995; Sturmi et al., 1998; Powell and Barber-Foss, 1999). In high school athletes, a cumulative effect of concussion was identified where repeat trauma significantly increased the severity of symptoms (Collins et al., 2002). Similarly, in collegiate football players, the magnitude and duration of symptoms (headache, nausea, confusion, fatigue, memory problems, attention deficits, and sleep disturbances) showed a cumulative effect after rmTBI (Guskiewicz et al., 2003). Several groups have shown that rmTBI produces longer lasting cognitive and motor deficits (De Beaumont et al., 2007; Omalu et al., 2010; Guskiewicz, 2011). No athlete should return to play while physical, cognitive, or behavioural symptoms are still present (McCrory et al., 2013).

1.5.2 Experimental rmTBI

Findings from laboratory research of brain injury suggest a state of enhanced vulnerability in response to injury, and that a second insult may exacerbate the damage (Jenkins et al., 1989; Hovda et al., 1991; Yoshino et al., 1991a). Mechanisms of this vulnerability have been attributed to cerebral ischemia (Jenkins et al., 1989), depressed cerebral oxidative metabolism (Hovda et al., 1991), and/or altered cerebral glucose utilization (Yoshino et al., 1991a). Additionally, rmTBI pathology has been attributed to increased axonal injury and associated degeneration, as well as other reactive changes identified by the presence of microglial activation (Laurer et al., 2001; Huh et al., 2007; Prins et al., 2010; Shitaka et al., 2011; Fujita et al., 2012). Brain atrophy and enlargement of the ventricles has also been identified after experimental repeat injuries (Maxwell, 2012; Wang et al., 2015).

In studies where a single mTBI does not produce significant neuropathological change, the same injury repeated within one to several days after the initial impact has been shown to cause pathological and/or behavioural abnormalities (Laurer et al., 2001; DeFord et al., 2002; Longhi et al., 2005; Huh et al., 2007; Friess et al., 2009; Prins et al., 2010; Shitaka et al., 2011). Using a controlled cortical impact (CCI) model, Laurer *et al.* (2001) identified pronounced motor deficits in response to rmTBI 24 hours apart with no impairment in single hit animals. BBB breakdown and axonal injury was exacerbated by rmTBI suggesting increased vulnerability to a second trauma for at least a day following the first injury (Laurer et al., 2001). Morris Water Maze (MWM) performance was impaired in a dose-dependent manner following one, two, or three mild lateral fluid percussion injury (FPI) in rats (DeRoss et al., 2002). Further evidence of decreased MWM performance has been found in mice, in addition to motor deficits and increased TAI. Deficits following CCI were most pronounced when the second injury was delivered three days after the first, and were greater than the single injury group (Longhi et al.,

2005). These findings were later expanded upon using MRI, which detected increased lesion volume compared to sham and mTBI when rmTBI were provided one or three days apart, but not after seven days (Huang et al., 2013). Huh and colleagues (2007) investigated the effect of rmTBI in immature rat pups. Enlarged ventricles and white matter atrophy was intensified following three impacts, while no pathology was observed after a single CCI (Huh et al., 2007). In neonatal piglets, moderate rotational non-impact injury delivered twice in 24 hours caused increased severity and mortality in comparison to two injuries seven days apart (Friess et al., 2009). Taken together, a second injury delivered between the temporal window of 24 – 72 hours exacerbated deficits (Laurer et al., 2001; Longhi et al., 2005; Friess et al., 2009; Huang et al., 2013) and repeat injuries appear to cause cumulative impairment (DeRoss et al., 2002; Huh et al., 2007).

1.6 TBI in juvenile populations

1.6.1 Clinical evidence

Epidemiological studies show that children are more likely to sustain a head injury than adults (Centers for Disease Control and Prevention, 2000; Rutland-Brown et al., 2006), and such injuries represent the leading cause of death and disability within the pediatric population (Kraus and Anderson, 1990; Levin et al., 1992). In Canada, there are approximately 18,000 hospitalizations annually due to TBI, of which 30% are children and youth (Canadian Institute for Health Information, 2006). Other population studies (USA), report annual rates of 180 per 100,000 children 15 years and younger that suffer a mild to severe TBI episode (Kraus, 1995). The primary cause of TBI varies with age, but motor vehicle accidents represent the leading cause of TBI in children (Keenan and Bratton, 2006). The majority of these head are closed-head injuries (CHI) (Filley et al., 1987) of mild severity (Yeates and Taylor, 2005). An early population study by Rivara investigated data from 197, 561 patients aged 18 years or younger

hospitalized for head injury. mTBI represented one-fifth of all head injuries, and was most prominent among school-age and teenage children. Nearly twice as many males sustained an injury than females (Rivara, 1984). Additionally, 30% of high-school students report at least one concussive event during their time at high-school (Segalowitz and Lawson, 1995). Sports-related injuries are the most prevalent cause of mTBI in children aged 10 – 19 (National Center for Injury Prevention and Control, 2003), and are of special consideration as they may result in rmTBI with cumulative effects (Collins et al., 1999; Matser et al., 1999).

Juvenile TBI is of special interest considering the human brain continues to mature into the early 20s (Romine and Reynolds, 2004; Sowell et al., 2004), and the developing nervous system is postulated to be more vulnerable to insults (Lewin et al., 1979; Luerssen et al., 1988; Levi et al., 1991). Children are known to have greater susceptibility to head injury. A study of children and adolescents who sustained a head injury before the age of eight displayed lasting cognitive deficits for 6+ years post injury (Verger et al., 2000). A year after hospitalization for TBI, children aged 5-15 displayed limitations in physical health, behavioural problems, and higher enrollment in special education programs (Greenspan and MacKenzie, 1994). Other long-term follow up studies with pediatric patients also demonstrate persistent neurocognitive deficits after TBI (Klonoff et al., 1977; Levin et al., 1982). Klonoff and colleagues (1993) contacted patients who were admitted to the hospital after sustaining a mild head injury during childhood, and approximately 23 years later, 31% reported persisting physical, intellectual, and emotional sequelae (Klonoff et al., 1993). In general, evidence from clinical studies show that children have more pronounced, longer lasting deficits in areas of memory, attention, and processing speed as compared to adults (Yeates, 2010). Such vulnerability may be that the biomechanical forces behind the injury are exaggerated as a result of the greater head to torso ratio and

relatively weaker neck compared to adults (Graham et al., 2014). Given the high incidence of TBI in children, and the long-lasting consequences of injury, it is important to further elucidate the pathophysiology and behavioural consequences of TBI in a juvenile pre-clinical model.

1.6.2 Pre-clinical evidence

Indeed, several groups have investigated the effect of TBI on the juvenile rodent. Depending on the severity of injury, a CHI delivered via weight drop induced persisting cognitive and motor deficits in post-natal day (PND) 17 rats (Adelson et al., 1997, 2000). In another modified weight drop model, juvenile (PND 30) male and female rats sustaining a single mTBI exhibited altered social play behaviour, impaired motor function and balance, learning and memory deficits, decreased executive function, depressive-like behaviour, and anxiety (Mychasiuk et al., 2014). The LFP model produced cognitive deficits after moderate severity injury in PND 28 animals. However, the same injury did not lead to significant differences in MWM performance in PND 17 animals (Prins and Hovda, 1998). LFP injury of mild, moderate, and severe severity all produced MWM deficits in PND 19 rats. These behavioural deficits were in the absence of significant cell death (Gurkoff et al., 2006). Similarly injured PND 19 rats did not benefit from rearing in an enriched environment as measured by MWM, suggesting TBI effects experience-dependent plasticity in the immature brain (Fineman et al., 2000; Giza et al., 2005). Prins and colleagues (1996) delivered LFP injuries of mild, moderate, and severe severity to PND 17, PND 28, and adult rats and measured intracranial pressure (ICP) and mean arterial blood pressure (MABP) post trauma. ICP and MABP increased with increasing severity, as did mortality rates. Immature rats exhibited the most pronounced hypotension in an age-dependent manner compared to adults, which was likely causative of higher mortality (Prins et al., 1996).

More recently, the impact of rmTBI in juvenile animals has also been evaluated. Juvenile (PND 20) rats were subjected to five mTBI over the course of five days using a modified weight drop model. MRI on post injury day (PID) 14 displayed Cortical atrophy and ventriculomegaly in the rmTBI animals (Goddeyne et al., 2015). Mannix and colleagues (2017) directly compared adult (4 months old) and adolescent (5 week old) mice after rmTBI. Both adolescent and adult repeat-injured mice presented increased impulsivity, impaired balance, and decreased spatial memory compared to sham animals. Deficits in balance and spatial memory were worse in adolescent mice (Mannix et al., 2017).

1.7 Animal models of TBI

Animal models are essential for studying the biomechanical, molecular, and cellular underpinnings of TBI that cannot be addressed with human patients in a clinical setting. Animal research also provides the opportunity for the development and testing of therapeutic interventions. Considering the heterogeneity of human TBI, numerous models are currently employed, but no one model can fully recapitulate all aspects of human TBI symptomology. However, animal models do offer controlled parameters of injury type and severity, age, sex, and genetic background. Rodents are the most commonly used in TBI research considering their relatively low operating cost, small size, and well-defined outcomes post injury. The best characterized rodent models of TBI include: weight-drop impact (Marmarou et al., 1994), FPI (Dixon et al., 1987), CCI (Lighthall, 1988; Dixon et al., 1991), and blast injury (Cernak et al., 1996; Leung et al., 2008) that will be discussed further.

1.7.1 Weight-drop injury

Injury is delivered by a free falling, guided weight onto an exposed skull (Marmarou et al., 1994; Morales et al., 2005). Different variations of the generalized weight-drop model

produce injury with or without craniotomy. CHI, or those without craniotomy, better represent the human condition (see Section 1.7.5). Severity of injury for weight-drop models is altered simply by adjusting the mass of weight or the height of the fall. The best characterized weight-drop model is that by Marmarou and colleagues (1994). This model uses a guided brass weight that impacts a stainless steel disc mounted to the skull of the animal. It is relatively inexpensive, easy to use, and can be delivered to the closed head to produce graded DAI that is similar to human TBI (Marmarou et al., 1994). Limitations include low reproducibility, potential for rebound injury and high mortality rate (Xiong et al., 2013).

1.7.2 Fluid percussion injury

Injury is delivered by a pendulum striking a piston of a reservoir of fluid that generates a fluid pressure wave to the intact dura through a craniotomy. The percussion force causes transient displacement and deformation of the brain tissue, and severity can be altered by varying the strength of pressure pulse generated (McIntosh et al., 1989). Subtypes of the FPI model vary based on location of the craniotomy, namely midline, parasagittal, and lateral based on distance from the midline. The lateral FPI is one of the most commonly used models in TBI animal research (Thompson et al., 2005). The FPI model offers high reproducibility and fine tuning of injury severity, but is limited by the need for anaesthesia, the use of craniotomy, and relatively high mortality rate (Xiong et al., 2013).

1.7.3 Controlled cortical impact injury

CCI injury is controlled by a pneumatic or electromagnetic impact device that drives an impactor onto the exposed dura through a craniotomy. The rigid impact causes deformation of the underlying cortex (Dixon et al., 1991). Injury severity can be tuned with degree of cortical deformation and impact velocity (Goodman et al., 1994; Saatman et al., 2006). More recently,

the CCI model has been characterized as a CHI model with no craniotomy. The CCI model offers high control of mechanical parameters, including time, velocity, and depth of impact. Limitations include the need for anaesthesia and the use of craniotomy, where applicable (Xiong et al., 2013).

1.7.4 Blast injury

Various blast injury models have been developed where a compression-driven shock tube is used to produce the injury in rodents (Cheng et al., 2010; Reneer et al., 2011; Wang et al., 2011; Risling and Davidsson, 2012) or in swine (Bauman et al., 2009; de Lanerolle et al., 2011). The goal of these blast models is to mimic those forces that many military personnel may be exposed to while on duty where the brain injury is typically indirect. Injury severity in blast models is based on the location of the animal relative to the shock tube. The orientation of the animal also affects the biomechanics of the injury, severity, and likely the mortality rate (Sundaramurthy et al., 2012). Blast injury models are unique and reflect the biomechanics associated with military TBI, however there is little consistency in the literature and reproducibility is limited (Xiong et al., 2013).

1.7.5 Limitations of current pre-clinical models

The vast majority of TBI animal models use anaesthesia during the delivery of injury. Isoflurane, is arguably, the most commonly used anaesthetic in experimental models of TBI, despite it rarely being used in clinical settings (Statler et al., 2006a). This represents a major caveat of TBI research as isoflurane has been shown to be neuroprotective by limiting of exicitotoxicity (Kimbro et al., 2000; Kudo et al., 2001), decreasing lipid peroxidation (Yurdakoc et al., 2008), and through pre-conditioning effects (Kapinya et al., 2002; Zhao and Zuo, 2004; Zheng and Zuo, 2004). Furthermore, isoflurane treatment prior to TBI improved performance on

the beam-walk test and in the MWM, and induced greater neuronal survival in the hippocampus (Statler et al., 2006b). Work by Statler and colleagues (2006) compared the effect of isoflurane with six clinically relevant sedative agents (siazepam, fentanyl, ketamine, morphine, pentobarbital, and propfol) following CCI in rats. Isoflurane was shown to afford the best cognitive recovery in the MWM and the highest hippocampal neuronal survival. The authors actually recommend isoflurane for its potential clinical benefits after TBI (Statler et al., 2006a). The use of anaesthetic agents in TBI research is a potential confound and may reduce the clinical relevance of the models.

Additionally, numerous rodent models of TBI include craniotomy in the injury paradigm (Taylor et al., 2008; Shultz et al., 2011, 2012a; Yu et al., 2012a; Almeida-Suhett et al., 2014; Cheng et al., 2014; Ghadiri et al., 2014; Arain et al., 2015). Like anaesthesia, craniotomy is not clinically relevant as most cases of human TBI are a CHI (Centers for Disease Control and Prevention, 2012). mTBI, by definition, occurs in the absence of skull fracture or gross pathology (McCrory et al., 2013), therefore injury models involving craniotomy cannot be, by definition a 'mild' injury. The craniotomy procedure itself activates pro-inflammatory agents that causes leakiness in exposed microvessels (Olesen, 1987). Futhermore, craniotomy can produce lesions detectable by MRI for 14 days after surgery, induce behavioral deficits, and elicit a pro-inflammatory response compared to naïve animals. Not only does this confound the interpretation of TBI models, but it also challenges the utility of the "sham" surgery as an appropriate control (Cole et al., 2011).

1.7.6 Awake Closed Head Injury

The use of anaesthesia and craniotomy in experimental TBI models produces confounds and limits the clinical relevance of pre-clinical studies. Petraglia and colleagues (2014)

previously developed a novel model of TBI that delivers closed-head impacts to *unanaesthetized* mice (Petraglia et al., 2014a). The present study has adapted the aforementioned model for rats with modifications. The Awake Closed Head Injury (ACHI) model, used herein, offers several benefits: (1) a restraint bag, rather than anaesthesia, is used to immobilize animals; (2) injuries are delivered to the closed head; (3) a helmet is used to target the injury; (4) the head is not fixed in a stereotaxic frame and; (5) the animal is positioned on a foam base prior to injury. The ACHI model, therefore, avoids the neuroprotective confounds of anaesthesia, does not cause distinct trauma with craniotomy, protects against skull fracture, and allows for rapid rotational acceleration of the brain. Taken together, the ACHI model is putatively a more relevant model of clinical TBI and offers greater translatability to the human condition.

1.8 Hippocampus

The hippocampus is a bilateral, seahorse shaped structure located within the medial temporal lobe that is composed of two subregions, namely the dentate gyrus (DG) and *cornu Ammonis* (CA). The CA, or hippocampus proper, is further divided into four sub-regions (CA1-4) (Bayer, 1980). The trisynaptic circuitry of the hippocampus involves successive unidirectional excitatory projections connecting the DG, CA1, and CA3 subregions (Andersen et al., 1971; Yeckel and Berger, 1990). The hippocampus is well established to play a crucial role in certain forms of learning and memory (Scoville and Milner, 1957; Bliss and Lomo, 1973; Morris et al., 1982; Teyler and DiScenna, 1985). The hippocampus is functionally distinct along its septo-temporal axis. The dorsal hippocampus is preferentially involved with spatial learning and memory, while the ventral hippocampus is implicated in affective behaviours such as depression and anxiety via its connection to the amygdala and limbic system (Moser and Moser, 1998; Bannerman et al., 2004).

1.8.1 Hippocampal vulnerability

Memory impairment has long been identified as the most common and persistent form of cognitive dysfunction associated with clinical mild closed-head injury (Gronwall and Wrightson, 1974, 1975; Rutherford, 1977; Rimel et al., 1981; McLean et al., 1983; Stuss et al., 1985; Binder, 1986). Moreover, the majority of mTBIs are sustained by primary impact to the frontal and temporal lobes of the brain, such that the hippocampus is commonly identified as a region of increased vulnerability (Bigler, 1999; Geddes et al., 2003; McCarthy, 2003; Tasker et al., 2005). The hippocampus sits adjacent to both these brain regions as well as the fluid filled ventricles, such that a head injury can expose the hippocampus to both the transmitted force and fluidic movement of cerebrospinal fluid (CSF) in the ventricles (Lindner *et al.*, 1998). The hippocampus is, therefore, an ideal structure to study and evaluate the effects of TBI.

Following TBI in adults, reductions in hippocampal volume have been detected with neuroimaging for months to years post-injury (Tomaiuolo et al., 2004; Kim et al., 2008). Moderate-to-severe TBI was further shown to cause significant volume loss in the hippocampus, and compared to the amgydala, globus pallidus, putamen, and caudate, as well as exterior cortical areas, the hippocampus was the most vulnerable structure to injury (Wilde et al., 2007). A longitudinal MRI study assessing mild to severe TBI observed significantly smaller hippocampal volumes and increased CSF volumes 10 years after injury, which was apparent across all levels of severity (Beauchamp et al., 2011). The juvenile brain also appears to be particularly susceptible to TBI. A five year follow up study by Tasker and colleagues (2005) identified reduced hippocampal volume following severe TBI during childhood (Tasker et al., 2005). These findings highlight the vulnerability of the hippocampus, including the juvenile brain, and suggest a putative link between the cognitive deficits seen post-injury and reduced hippocampal volume.

Experimental TBI models have generated complimentary findings to that of the clinical population. Reduction in hippocampal volume and atrophy have been reported following TBI in rodents (Tate and Bigler, 2000; Bramlett and Dietrich, 2002; Creed et al., 2011). In a non-impact rotational acceleration model, miniature swine displayed significant neuronal damage primarily in CA1 and CA3 hippocampal sub-regions (Smith et al., 1997). Interestingly, rats subjected to mild or moderate TBI displayed no CA1 neuronal death, but displayed radial-arm maze deficits for five and 15 days post injury (Lyeth et al., 1990). The DG is also susceptible to TBI induced damage. FPI caused marked ipsilateral neuronal loss and lesser, yet significant loss in the contralateral DG. In addition to bilateral hippocampal neuron loss, DG physiology was altered (Lowenstein et al., 1992). Our laboratory has previously identified sex differences in hippocampal function following weight drop injury delivered to juvenile rats. Long-term potentiation (LTP), a form of synaptic plasticity, was significantly reduced in the female ipsilateral DG 24 hours after mTBI and remained that way for 28 days following injury. In the male rat, LTP deficits were pronounced in both the ipsilateral DG and CA1 hippocampal sub-regions at seven days post injury, but did not persist (White et al., 2016). Such hippocampal atrophy and dysfunction may underlie the memory impairment observed in experimental models of TBI, and be of potential relevance for clinical head injury (Levin, 1985).

Cognitive impairment has also been demonstrated following experimental brain injury. The MWM is the most widely used behavioural test of hippocampal-dependent spatial learning and memory (Morris et al., 1982). Decreased MWM performance has been demonstrated using a variety of TBI models including weight-drop impact (Zohar et al., 2003, 2011; Nichols et al., 2016), CCI (Creed et al., 2011; Washington et al., 2012; Johnson et al., 2013; Petraglia et al., 2014a), FPI (Shultz et al., 2011, 2012a), and blast injury (Turner et al., 2015). Other tests of

hippocampal function that have shown deficits post-TBI include the novel object recognition test (Prins et al., 2010), Barnes maze (Kwon et al., 2011), the metric change and temporal order tasks (Gurkoff et al., 2013). Evidence of hippocampal impairment observed in experimental animals parallels that seen in humans. All things considered, one can speculate that damage to the hippocampus following mTBI may underlie some of the cognitive and affective symptoms characteristic of mTBI in the clinical setting.

1.9 Neurobehavioural sequelae and ethology

Cognitive, behavioural, physical, emotional, and social problems are common and significant causes of disability and trauma for TBI patients (Gordon et al., 1998; Hibbard et al., 1998b). Collectively, the term neurobehavioural sequelae refers to the development of multifactorial symptoms post-injury. The neurobehavioural sequelae of mTBI can be broken down into two categories: somatic and/or neuropsychiatric. The somatic symptoms were mentioned previously in Section 1.4 (headaches, dizziness, nausea, fatigue, *etc*). Neuropsychiatric symptoms include cognitive (attention, memory, and executive function deficits) and behavioural changes (affective disorders, aggression, irritability, anhedonia, and apathy). In some cases, previous psychiatric disorders may be exacerbated by TBI (Riggio, 2011). While the majority of mTBI cases will show full recovery (Dikmen et al., 2001), a subset of patients will develop persistent post-traumatic neuropsychiatric symptoms and disability (Hessen et al., 2007). Approximately 30-80% of patients with mild to moderate TBI experience NBS that may persist up to three months post-injury (Carroll et al., 2004). In 15% of mTBI patients, clinical symptoms persist beyond three months and can contribute to long-term problems that can significantly impair the quality of life of these individuals (Ruff et al., 1996; Ponsford et al., 2002; Lundin et al., 2006).

Behavioural testing in pre-clinical models has been used to study post-TBI-like symptoms seen in the human population. Animal models facilitate further investigation of the underlying mechanisms of TBI symptomology and aid in the search for therapeutic interventions. Again, no single model can fully recapitulate all aspects of human TBI symptomology, especially considering the complex nature of neuropsychiatric disorders. Nonetheless, several behavioural tests have been developed to examine depression, anxiety, impulsivity, and motor impairment in rodent models of TBI.

1.9.1 Depression

Depression is the most prominent psychological disturbance resulting from TBI, which is reported in 6% of mTBI cases (Rutherford, 1977) and up to 77% in more severe TBI cases (Jorge et al., 1993). Typically occurring within the first year after injury (Alderfer et al., 2005), the primary symptoms of depression after TBI are persistent sadness, negativity, loss of pleasure, hopelessness, and suicidality (Vaishnavi et al., 2009). Post-traumatic depression may increase the prevalence and perceived severity of self-reported post-injury symptoms such as headache, dizziness, and blurred vision (Fann et al., 1995; Rapoport et al., 2003). Moreover, increased aggression, anger, suicidal thoughts, and cognitive dysfunction have been linked to TBI-induced depression (Hibbard et al., 1998a; Fann et al., 2001; Rapoport et al., 2005). Impaired ability to perform normal daily tasks and other associated psychosocial changes as a result of injury likely exacerbate depressive symptoms (Pagulayan et al., 2008). Evidence from retired football players indicates that the likelihood of experiencing depression-like symptoms is increased nearly three-fold with rmTBI (Guskiewicz et al., 2007).

Considering the prevalence of depression following TBI in humans, the Porsolt Forced Swim Test (FST) is commonly implemented in TBI research as a test of depressive-like

behaviour (Porsolt et al., 1978). In rodent models, the effect of TBI on depression is controversial. Depression-like symptoms have been observed in mice following weight drop impact (Milman et al., 2005), and CCI (Washington et al., 2012; Petraglia et al., 2014a), as well as in rats following CCI (Taylor et al., 2006). In contrast, several studies did not observe any differences between sham and injured animals (Jones et al., 2008a; Shultz et al., 2011, 2015; Cheng et al., 2014; Mychasiuk et al., 2015; Nichols et al., 2016). Previous work by Shultz and colleagues showed that three repeat mild FPI in five days, or five injuries every five days was sufficient to induce depressive-like symptoms that were not evident after a single mild injury (Shultz et al., 2012a; Tan et al., 2016).

1.9.2 Anxiety

Anxiety develops in 10-70% of patients that have sustained a mTBI (Mooney and Speed, 2001; Rao and Lyketsos, 2002; Moore et al., 2006; Silver et al., 2009; Vaishnavi et al., 2009). Commonly identified anxiety symptoms post-TBI include general anxiety, fearfulness, uneasiness, stress, social withdrawal, sensitivity, and anxious dreams (Rao and Lyketsos, 2002). Post-traumatic stress disorder (PTSD) falls under the umbrella of anxiety disorders, and is frequently experienced by TBI patients. PTSD is defined as anxiety of re-experiencing trauma, emotional numbing, hypervigilance, and avoidant behaviour (Vaishnavi et al., 2009).

The open-field test is commonly used in TBI research to assay anxiety-like behaviour. In rodents, TBI was found to cause anxiety-like symptoms 24 hours (Kwon et al., 2011), seven days (Almeida-Suhett et al., 2014), 10 days (Yu et al., 2012a), one month and three months post injury (Jones et al., 2008a). Conversely, other studies have shown no anxiety-like behaviour in the open-field test (Washington et al., 2012; Cheng et al., 2014; Iliff et al., 2014; Shultz et al., 2015; Tan et al., 2016).

1.9.3 Impulsivity and risk-taking behaviour

Personality changes after TBI may include aggression, irritability, emotional lability, apathy or impulsivity (Greve et al., 2001; Tateno et al., 2003). Various clinical studies have identified increased impulsivity or risk-taking behaviours following mTBI (Rapoport et al., 2003; Salmond et al., 2005; Tellier et al., 2009; Newcombe et al., 2011). General impulsivity can evolve into more serious problematic behaviours such as substance abuse (Parry-Jones et al., 2006), aggression (Vaishnavi et al., 2009), and even suicidality (Brenner et al., 2009).

The elevated-plus maze (EPM) has previously been employed to assess impulsivity following experimental TBI, again with some inconsistency in the literature. Increased time in the open arms, a measure of impulsivity or risk-taking behaviour, was identified following TBI (Pandey et al., 2009; Shultz et al., 2011; Washington et al., 2012; Johnson et al., 2013; Logsdon et al., 2014; Turner et al., 2015). Other groups show no differences between TBI and sham animals in the EPM (Cheng et al., 2014; Arain et al., 2015; Shultz et al., 2015; Nichols et al., 2016; Tan et al., 2016), while some actually show decreased time in the open arms (Jones et al., 2008a; Kwon et al., 2011; Mouzon et al., 2014; Mychasiuk et al., 2015).

1.9.4 Motor coordination deficits

In addition to the aforementioned neuropsychiatric symptoms following TBI, motor dysfunction and balance deficits post-injury are also well-defined. Even mTBI can result in problems with balance and equilibrium deficits as seen in athletes (Guskiewicz, 2011). Additional and related symptoms include dizziness, vertigo, lightheadedness, photophobia, tinnitus, and blurred vision (Ingersoll and Armstrong, 1992; Wöber et al., 1993; Geurts et al., 1996; Greenwald et al., 2001; Campbell and Parry, 2005; Kaufman et al., 2006; Rinne et al., 2006). Sports-related concussions lead to balance problems 30% of the time (Guskiewicz et al.,

2000), and deficits have been shown to resolve within 3-10 days post-injury (Guskiewicz et al., 1997, 2001; McCrea et al., 2003; Peterson et al., 2003).

The Rota-Rod was first implemented in TBI research in 1994, and was found to be a sensitive and effective test of mTBI-induced motor dysfunction (Hamm et al., 1994). The Rota-Rod has since been implemented in TBI research on several occasions. TBI animals consistently show decreased performance on this task compared to shams in studies using mice (Longhi et al., 2005; Kane et al., 2012; Yu et al., 2012a; Yang et al., 2013b; Mannix et al., 2017) and rats (Yan et al., 2013; Maegele et al., 2015). No differences in Rota-Rod performance have also been reported (Iliff et al., 2014; Luo et al., 2014; Mouzon et al., 2014).

1.9.5 Considerations for TBI behavioural research

It is evident that there are discrepancies in the TBI literature in regard to behavioural deficits post-injury. Such inconsistency stems, in part, from shortcomings in behavioural research in general: different laboratories employ different conditions, protocols, apparatus, as well as differences from the species, strain, sex, and age of animal used. Considering TBI research specifically, different outcomes may arise from the model of TBI used, the use of anaesthesia, craniotomy surgery, the severity of injury, location of injury, and the time post-injury when the testing is performed. The standardization of behavioural techniques and protocols, as well as universal models of TBI would greatly benefit the advancement of the field and assist in the elucidation of the effect of TBI on depression, anxiety-like behaviour, impulsivity/risk-taking behaviour, and motor deficits.

1.10 Chronic Traumatic Encephalopathy

The phenomenon of multiple concussions was first described by Harrison Martland (1928), then coined as “punch drunk syndrome.” He used the term to describe the mannerisms

of boxers who had sustained repeated blows to the head, such as ataxia, confusion, and altered speech (Martland, 1928). Millspaugh later defined the condition as 'dementia pugilistica' (Millspaugh, 1937). Additional evidence and case studies describing the negative consequences of repeat injury in boxing followed, and further identified the potentially chronic and progressive nature of an underlying neuropsychiatric disorder (Critchley, 1957; Corsellis and Brierley, 1959; Payne, 1968). Seminal work by Corsellis and colleagues (1973) investigated the pathological features of dementia pugilistica in the brains of 15 former boxers. They reported cerebral atrophy, enlargement of the ventricles, cerebellar scarring, thinning of the corpus callosum and neurofibrillary degeneration using cresyl violet (Corsellis et al., 1973). However, parallel studies recognized that the condition was not limited only to boxing, such that the term chronic traumatic encephalopathy (CTE), first introduced by Critchley (Critchley, 1949), eventually became the preferred classification.

CTE is a progressive neurodegenerative disease characterized by hyper-phosphorylated tau deposits throughout the brain in the form of neurofibrillary tangles (NFTs) (McKee et al., 2009, 2013). Patient's suffer from memory impairments, executive function difficulties, depression, suicidality, substance abuse, and ultimately, death (Stern et al., 2011; Mez et al., 2013). In addition to boxers, numerous case studies have identified cases of CTE in American football (Omalu et al., 2005, 2006, 2010), military veterans exposed to blast exposure (Omalu et al., 2011b; Goldstein et al., 2012; McKee and Robinson, 2014), rugby (Stewart et al., 2016), soccer, baseball, and ice hockey (McKee et al., 2009, 2013, 2014). At this time, the disease is only diagnosed during autopsy (Saulle and Greenwald, 2012), yet numerous case studies of post-mortem brain tissue, support CTE as the long-term consequence of rmTBI (Corsellis and Brierley, 1959; Corsellis et al., 1973; Geddes et al., 1999; Omalu et al., 2005, 2006; McKee et

al., 2009; Saing et al., 2012). However, it remains unclear whether a single injury is sufficient to produce this neuropathology or how many rmTBI are required. Currently, the incidence and prevalence of CTE remains to be determined, and warrants further investigation.

1.11 Tau protein

Tau protein is a key microtubule-associated protein (MAP) that is abundant in the central nervous system where it is primarily expressed in the axons of neurons (Binder et al., 1985; Trojanowski et al., 1989). In fact, tau accounts for more than 80% of the total MAPs in the brain (Witman et al., 1976). Functionally, tau binds microtubules (MT) to promote assembly and elongation, and acts to stabilize existing MTs (Weingarten et al., 1975; Witman et al., 1976). The binding stoichiometry of tau protein is approximately one tau for every two tubulin dimers (Mandelkow et al., 1996). As an essential MAP, tau is involved in axonal growth through modulation of the extent and rate of MT assembly, and establishes a stable, yet dynamic state (Trinczek et al., 1995).

1.11.1 Tau structure

The human tau gene is located on chromosome 17 at band position 17q21 (Neve et al., 1986). The tau primary transcript contains 16 exons, of which, exon 2, 3, and 10 are alternatively spliced and are adult brain specific (Andreadis et al., 1992). Via mRNA alternative splicing, six different tau protein isoforms exist in the human brain that range in length from 352-411 amino acid residues (Goedert et al., 1989; Himmler et al., 1989; Andreadis et al., 1992, 1995). The isoforms vary, not only in amino acid length, but also in the number of amino-terminal inserts (0, 1, or 2) and the number of microtubule-binding repeats (3 or 4). Splice variants that retain exon 10 have four microtubule-binding repeats (4R-tau) and those without exon 10 contain three (3R-Tau). In the normal adult brain, 4R-Tau and 3R-Tau exist in equal

concentrations (D'Souza and Schellenberg, 2005; Donahue et al., 2007). However, 4R-Tau isoforms are more easily phosphorylated by kinases (Alonso et al., 2004), bind more effectively to MTs (Dayanandan et al., 1999), and are more commonly associated with tau-linked neurodegeneration (Wang and Liu, 2008).

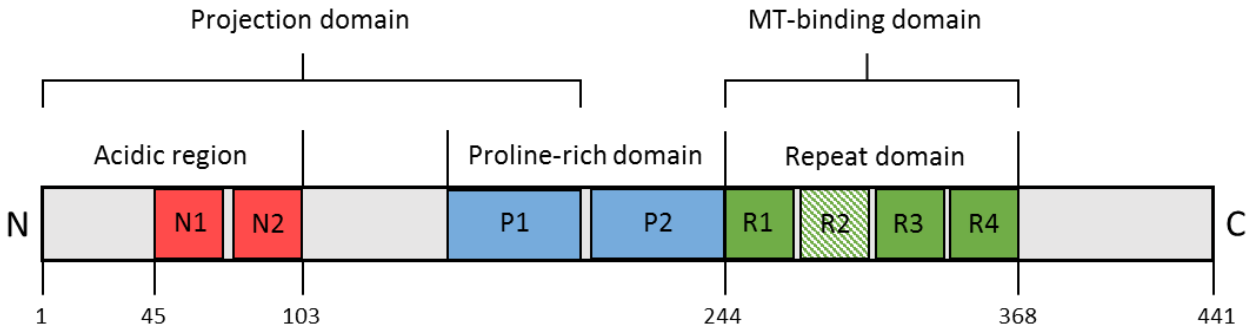

Figure 2. Tau protein structural features. The structural domains of the longest isoform of the human tau protein, Tau_{441} (2N4R), on a linear diagram. Eight distinct domains exist: two N-terminal domains (N1 and N2), two proline-rich domains (P1 and P2), and four repeat, microtubule-binding domains (R1-4). N1, N2, and R2 represent exon 2, 3, and 10, respectively. Image adapted from (Mandelkow et al., 1996; Pedersen and Sigurdsson, 2017).

Tau protein in the brain can be structurally divided into two primary functional regions: the projection domain and the MT-binding domain, which constitute 2/3 and 1/3 of the molecule, respectively (**Figure 2**). The projection domain is thought to regulate spacing between microtubules within the axon (Chen et al., 1992), and interact with other cytoskeletal proteins, thereby contributing to normal architecture (Hirokawa et al., 1988). The MT-binding domain contains three (3R-Tau) or four (4R-Tau) repeat sequences, which can bind to MTs and afford tau its primary biological function (Trinczek et al., 1995). The middle region of tau is a proline-rich domain (**Figure 2**) that contains several Serine-Pro or Threonine-Pro motifs, which are the targets of multiple proline-directed kinases (Biernat et al., 1992; Augustinack et al., 2002; Wang and Mandelkow, 2015).

1.11.2 Regulation of tau protein

Tau is a phosphoprotein that contains 1-3 mole of phosphate per mole of tau protein in the healthy adult brain (Ksiezak-Reding et al., 1992; Köpke et al., 1993; Watanabe et al., 1993). Phosphorylation of tau is crucial for its normal functioning. Regulated phosphorylation of tau determines its affinity for microtubule binding (Grundke-Iqbal et al., 1986b; Drechsel et al., 1992; Bramblett et al., 1993; Harada et al., 1994; Seubert et al., 1995). Phosphorylation at threonine-231 and serine-262, specifically, results in decreased MT affinity (Lindwall and Cole, 1984; Mandelkow et al., 1996), which allows for regulation of microtubule length (Ahmad et al., 1999; Baas and Qiang, 2005; Qiang et al., 2006) and increased molecular transport (Baas et al., 2005). More than ten serine/threonine kinases that phosphorylate tau have been identified with *in vitro* studies. The primary tau kinases include glycogen synthase kinase-3 (GSK-3; see section 1.12) (Hanger et al., 1992; Mandelkow et al., 1992a; Yamaguchi et al., 1996; Pei et al., 1997, 1999), cyclin-dependent kinase 5 (cdk5) (Baumann et al., 1993; Kobayashi et al., 1993; Paudel et al., 1993; Noble et al., 2003), as well as extracellular protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) (Drewes et al., 1992; Wang et al., 2007; Wang and Liu, 2008). Serine/threonine protein phosphatase 2A (PP2A) is the primary tau phosphatase (Wang et al., 1995; Liu et al., 2005, 2006). Tau protein phosphorylation is tightly regulated, such that several kinases and phosphatases likely function in parallel (Sengupta et al., 1998; Zheng-Fischhöfer et al., 1998; Jicha et al., 1999). Moreover, the rate and amount of tau phosphorylation is further regulated by the priming, or pre-phosphorylation, required by certain kinases to act on tau (Singh et al., 1995; Sengupta et al., 1997).

1.11.3 Tau hyper-phosphorylation

Despite the variety of factors involved in the phosphorylation-dependent regulation of tau protein, aberrant phosphorylation can occur. Abnormal or hyper-phosphorylation of tau causes reduced affinity and subsequent dissociation from MTs, which may result in destabilization of MTs and impaired anterograde axonal transport (Li et al., 2007; Cowan et al., 2010). The leading cause of tau hyper-phosphorylation is the overexpression of kinases and the down regulation of phosphatases (Zhang et al., 2009). Among the various kinases and phosphatases, GSK-3β and PP2A are the most implicated (Tian and Wang, 2002; Avila et al., 2004). Tau is a natively unfolded and disordered protein (Iqbal et al., 2009). However, hyper-phosphorylation promotes self-assembly into a folded conformation and increases the propensity to aggregate (Alonso et al., 1996, 2001; Jeganathan et al., 2008). These insoluble aggregates of tau sequester normal tau and other MAPs, further destabilizing MTs, inhibiting MT assembly and impairing intracellular trafficking (Alonso et al., 1996, 1997; Salehi et al., 2003; Iqbal et al., 2008). Further aggregation of tau results in the formation of paired helical filaments (PHF) and subsequently NFTs, which are neurotoxic (Li et al., 2007; Cowan et al., 2010) and characteristic of several neurodegenerative disorders, termed tauopathies (Mandelkow and Mandelkow, 2012; Kumar et al., 2014).

Neurodegenerative disorders characterized by tau NFTs are termed tauopathies (Lee et al., 2001). Alzheimer's disease (AD) is most well-defined tauopathy. In 1986, tau was first discovered to be abnormally hyper-phosphorylated in an AD brain and to be the principal protein component of NFTs (Grundke-Iqbal et al., 1986a, 1986b; Iqbal et al., 1986). In AD brains, the phosphorylation level of tau is 3-4 fold higher than that of the tau isolated from age-matched control brains (Khatoon et al., 1992, 1994; Mandelkow et al., 1996) and more than 30 serine/threonine residues are phosphorylated (Hanger et al., 1998). Additional tauopathies

include Pick's disease, progressive supranuclear palsy, agyrophilic grain disease, amyotrophic lateral sclerosis, Parkinsonism, corticobasal degeneration, and CTE (Lee et al., 2001; Rajput et al., 2006; Santpere and Ferrer, 2009; Omalu et al., 2011a).

1.11.4 Tau protein and TBI

Tau pathology following brain trauma has been investigated experimentally on several occasions, especially in the last decade. Numerous animal models, including weight drop, CCI, FPI, and blast injury, are used to investigate tau pathology following either single or repeat injuries. Indeed, the majority of models characterized within the last several years deliver repeat impacts and assess changes to tau phosphorylation considering the increased focus on CTE research in humans (Section 1.10). A variety of pre-clinical models have since been developed to specifically model CTE in rodents (Goldstein et al., 2012; Petraglia et al., 2014a; Turner et al., 2015; Zhang et al., 2015; Lucke-wold et al., 2016). An extensive review of recent TBI literature investigating tau protein is included in **Table 1.** The studies are presented chronologically and organized by single or repeat injury. See Chapter 4 for a greater discussion of the current study in comparison to previously presented literature.

Table 1. TBI models investigating tau pathology

Authors	**Animal** Species/Sex/ Age	**TBI model**	**Injury**	**Anaesthesia**	**Detection technique**	**Time post-injury**	**Results**
Hoshino *et al.* 1998	S. Dawley rat Male 3 months	Lateral FPI (open)	Single	Chloral hydrate	IHC (coronal sections)	2, 4, 6 months	pTau immunoreactivity @ 6 months post
Smith *et al.*, 1999	Miniature pig Male/Female 4 month	Rotational acc. (CHI)	Single	Midazolam, isoflurane	IHC (coronal sections)	1, 3, 7, 10 days	Tau detected throughout brain at 3-10 days
Genis *et al.*, 2000	APOE-KO/ C57BL6 mice Male 4 months	Weight drop (CHI)	Single	*Not stated*	WB (Fore-brain)	4 , 24 h, 7 days	Increased pTau @ 4 hr post, baseline by 24 hr

Gabbita *et al.*, 2005	S. Dawley rat Male 8-10 weeks	CCI (open)	Single	Isoflurane	ELISA, WB (HPC, CTX)	6, 24 h, 2, 3, 7 days	Elevated cTau 6h to 7 days post
Liliang *et al.*, 2010	S. Dawley rat Male ~12 weeks	Weight drop (open)	Single	Isoflurane	ELISA, WB (serum)	1, 6, 24 h, 2, 7 days	Elevated tau at 1 and 6 h
Tran *et al.*, 2011a	3xTg-AD mice Male/Female 5-7 months	CCI (open)	Single	Isoflurane	WB (HPC)	24 h	Increased pTau in ipsilateral hemisphere
Liu *et al.*, 2011	S. Dawley Male 2-3 months	CCI (open)	Single	Isoflurane	WB (CTX, HPC)	2, 6, 24 h, 2, 3, 4, 7, 14 days	Accumulation of tau breakdown products, peak around 2 days
Goldstein *et al.*, 2012	C57BL6 mice Male 2.5 months	Blast (CHI)	Single	Ketamine, xylazine, buprenor-phine	WB (CTX)	14 days	Elevated pTau, increased pTau/Tau ratio
Kovesdi *et al.*, 2012	S. Dawley Male ~ 8-9 weeks	Blast (CHI)	Single	Isoflurane	RPPM (PFC, HPC, amygdala)	2.5 months	Elevated tau in all regions
Tran *et al.*, 2012	3xTg-AD mice Male/Female 5-7 months	CCI (open)	Single	Isoflurane	WB (HPC)	24 h	JNK inhibitor reduced tau
Yu *et al.*, 2012	C57BL6 mice Male 7 weeks	CCI (open)	Single	Isoflurane	ELISA, IHC (coronal sections)	3 days	Increased pTau; reduced by lithium
Rostrami *et al.*, 2012	S. Dawley Male ~10-15 weeks	Rotational acc. (open)	Single	Midazolam, fentanyl, fluanisone	RPPM (serum)	1, 3, 14 days	Increased tau at all time-points
Huber *et al.*, 2013	C57BL6 mice Male 3-4 months	Blast (CHI)	Single	Isoflurane	WB, IHC (HPC, CTX, CB)	24 h, 1 month	WB: no change IHC: increased pTau at 1 month
Hawkins *et al.*, 2013	S. Dawley Male ~15+ weeks	FPI (open)	Single	Isoflurane	ELISA, WB	4, 24 h, 14 days	Increased pTau at all time-points
Iliff *et al.*, 2014	C57BL6 mice Male 8-12 weeks	CCI (CHI)	Single	Isoflurane	WB (brain)	28 days	Increased pS396 in ipsi- and contralateral
Perez-Polo *et al.*, 2015	S. Dawley Male ~12 weeks	Blast (CHI)	Single	Isoflurane	IHC (HPC, CTX)	6 h, 1 month	pTau immune-reactivity at 6 h to 1 month

Kane *et al.*, 2012	C57BL6 mice Male *Not stated*	Weight drop (CHI)	1/day for 5 days	Isoflurane	WB (CTX)	1 month	Increased pTau after rmTBI
Arun *et al.*, 2013	C57BL6 mice Male 8-10 weeks	Blast (CHI)	3 injuries	Isoflurane	WB (CB, serum)	6, 24 h	Decrease total tau at 6 h, increase at 24 h
Ojo *et al*., 2013	hTau mice Male/Female 18 months	CCI (CHI)	Single, 5 hit in 9 days	Isoflurane	IHC (coronal sections)	21 days	Increase pTau after rmTBI, not single injury
Luo *et al*., 2014	C57BL6 mice Male 3 months	CCI (CHI)	3 mTBI	Isoflurane	IHC (coronal sections)	6 months	pTau immunoreactivity @ 6 months post
Petraglia *et al.*, 2014	C57BL6 mice Male 12 weeks	CCI (CHI)	Single, 42 hits in 6 days	Awake model; no anaesthesia	IHC (HPC, CTX, amygdala)	7 days, 1, 6 months	Single: increased pTau at 7 days and 1 month rmTBI: increased pTau 7 days – 6 months
Zhang *et al.*, 2015	C57BL6 mice Male 6-10 weeks	CCI (CHI)	1/day for 3 days	Tribomo-ethanol	WB (HPC, CTX)	8 days, 1 month	Elevated pTau at 8 days and 1 month
Lucke-Wold *et al.*, 2016	S. Dawley Male 2-3 months	Blast (CHI)	Single, 6 hits in 2 weeks	Isoflurane	IHC, WB	24 h, 2 weeks	Increased pTau 2 weeks post injury

CB, cerebellum; CCI, controlled cortical impact; CHI, closed head injury; CTX, cortex; ELISA, enzyme-linked immunosorbent assay; FPI, fluid percussion injury; HPC, hippocampus; IHC, immunohistochemistry; PFC, prefrontal cortex; RPPM, Reverse Phase Protein Microarray; WB, western blot

1.12 Glycogen Synthase Kinase-3

Glycogen synthase kinase (GSK)-3 is a ubiquitously expressed proline-directed serine/threonine kinase. It was first identified as an enzymatic regulator of glucose metabolism (Woodgett and Cohen, 1984; Plyte et al., 1992), but has since been implicated in the regulation of cell division (Diehl et al., 1998), cell signaling (Guha et al., 2011), differentiation, proliferation, and growth (Force and Woodgett, 2009; Shin et al., 2011), as well as apoptosis (Watcharasit et al., 2003). In addition to the glycogen synthase enzyme of glucose synthesis, GSK-3 is now known to target a wide range of substrates. Included in this spectrum are transcription factors such as c-Jun, c-Myc, c-Myb, β-catenin, nuclear factor kappa-light-chain-

enhancer of activated B cells (NF-κB), nuclear factors of activated T-cells (NFAT), zinc-finger transcription factor (Snail), and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Zhou et al., 2004; Götschel et al., 2008). Additional substrates include ATP-citrate lyase, cyclic-AMP-dependent protein kinase, the translation factor eIF-2B, and tau protein (Plyte et al., 1992; Welsh and Proud, 1993; Fiol et al., 1994).

1.12.1 GSK-3 isoforms

Mammalian GSK-3 exists as two isoforms, α and β. The α isoform (51 kDa) is slightly longer than the β isoform (47 kDa) as it contains a glycine-rich extension at its amino-terminal end (Woodgett, 1990). Each are encoded by a distinct gene, yet display 85% amino acid homology (Welsh et al., 1996). Both isoforms are expressed throughout the brain with highest levels of expression seen in the hippocampus, cerebral cortex, and the cerebellum. Overall, relatively greater amounts of GSK-3β are expressed (Lau et al., 1999; Yao et al., 2002). However, GSK-3α is also found in the heart, liver, lungs, skeletal muscle and spleen (Yao et al., 2002). The α and β isoforms have a highly conserved (97%) catalytic kinase domain and share many similar substrates, yet remain pharmacologically distinct (Kockeritz et al., 2006). GSK-3β knockout mice are embryonically lethal, while the GSK-3α knockout mice are viable proving that the two isoforms are not interchangeable (Hoeflich et al., 2000).

1.12.2 GSK-3 regulation

Two key functional domains of GSK-3 have been identified. They include (1) a primed-substrate binding domain that recruits pre-phosphorylated substrates, and (2) a kinase domain for additional phosphorylation of the substrate (Frame et al., 2001; Dajani et al., 2003). Inhibitory serine phosphorylation is the primary mechanism that regulates activity of GSK-3. Phosphorylation of GSK-3α at serine-21 (Ser21) or GSK-3β at serine-9 (Ser9) causes the N-

terminal tail of GSK-3 to act as a pseudosubstrate, thereby blocking the binding pocket to other primed-substrates (Woodgett, 1990; Frame et al., 2001; Moore et al., 2013). This inhibition by phosphorylation is unconventional, but necessary considering both GSK-3α and GSK-3β are constitutively active kinases (Woodgett, 1990).

Multiple signaling pathways regulate GSK-3 activity. Akt, or protein kinase B (PKB), is the most well-defined upstream kinase (Cross et al., 1995), in addition to protein kinase A (PKA) and protein kinase C (PKC) (Jope and Johnson, 2004). As such, any upstream factors to these kinases can also indirectly inhibit GSK-3 activity. GSK-3 may also be activated by phosphorylation at Tyrosine-279 on GSK-3α and Tyrosine-16 on GSK-3β (Kockeritz et al., 2006). Despite this, deactivation via phosphorylation has a greater influence on activity considering the constitutively active nature of the enzyme and that activation sites can also undergo autophosphorylation (Cole et al., 2004). Further means of GSK-3 regulation includes dephosphorylation of inhibitory sites by protein phosphatase 1 (PP1), PP2A, and protein phosphatase 2B (PP2B, calcineurin) (Peineau et al., 2007; Kim et al., 2009; Hernández et al., 2010). Quantifying the relative phosphorylation of Ser21 or Ser9 is a common method of analyzing the level of inhibition, and consequently the level of activity, of GSK-3 under different experimental conditions.

1.12.3 GSK-3β in tau pathology

Cell culture and *in vitro* studies have strongly characterized a link between GSK-3 and tau phosphorylation. Research has shown that both GSK-3α and GSK-3β can phosphorylate tau at several epitopes that are found to be hyper-phosphorylated in AD brains (Hanger et al., 1992; Mandelkow et al., 1992b; Lovestone et al., 1994; Sperber et al., 1995; Li and Paudel, 2006). However, is it GSK-3β, specifically, that has been identified as a primary tau kinase and

researched extensively. GSK-3β has been shown to be associated with normal MT-bound tau (Sun et al., 2002), as well as with hyper-phosphorylated tau deposits in the AD brain (Yamaguchi et al., 1996; Ferrer et al., 2002). *In vivo* studies where GSK-3β is overexpressed in animal models promotes tau protein phosphorylation (Brownlees et al., 1997; Spittaels et al., 2000; Lucas et al., 2001; Engel et al., 2008) and accelerates tau-induced neurodegeneration (Spittaels et al., 2000; Lucas et al., 2001; Engel et al., 2006a, 2006b). Tau phosphorylation is reduced in the presence of GSK-3β inhibitors or upstream Akt inhibitors (Takahashi et al., 1999; Leclerc et al., 2001; Shi et al., 2008; Greco et al., 2009; Zhang et al., 2011). Altered GSK-3β kinase activity is proposed to interfere with normal tau phosphorylation thereby altering tau functioning. This likely leads to disruption of MT stability (Lovestone et al., 1996; Sang et al., 2001), MT assembly (Utton et al., 1997), and MT-dependent axonal transportation (Tatebayashi et al., 2004; Cuchillo-Ibanez et al., 2008).

1.12.4 GSK-3β after TBI

GSK-3β dysregulation is linked to pathogenesis of several disorders, including cancer (Sokolosky et al., 2014; Azoulay-Alfaguter et al., 2015), diabetes (Gao et al., 2011), neuroinflammation (Li et al., 2013), and neurodegenerative diseases such as Alzheimer's (Lei et al., 2011; Avrahami et al., 2013) and Parkinson's Disease (Kwok et al., 2005; Duka et al., 2009; Nagao and Hayashi, 2009). Moreover, GSK-3β has been shown to cause cell death via activation of pro-apoptotic signals (Pap and Cooper, 1998), while inhibition of GSK-3β has been linked to neuroprotective outcomes (Cross et al., 2001; Li et al., 2002; Hongisto et al., 2003). Considering the role of GSK-3β in neurodegenerative pathophysiology, changes to GSK-3β activity has been investigated following TBI. Previous work has shown that TBI induces a

significant increase in GSK-3β phosphorylation at Ser9 (Shapira et al., 2007; Dash et al., 2011; Yu et al., 2012a; Zhang et al., 2015).

1.13 Akt

Akt, synonymous with PKB, is a serine/threonine kinase involved in a plethora of intracellular signaling pathways. This key regulatory protein exists as three isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) in mammalian cells (Chan et al., 1999). All three genes share greater than 85% sequence homology resulting in the same structural protein organization. As such, the Akt isoforms are assumed to have similar substrate specificity. The isoforms are ubiquitously expressed with Akt1 being the predominant isoform in most tissues (Kandel and Hay, 1999).

1.13.1 Akt Regulation

Akt is a key downstream factor of the phophoinositide 3-kinase (PI3K) pathway. PI3K is stimulated by a variety of growth factors and cytokines [For Review: (Vanhaesebroeck et al., 2010; Ghigo et al., 2012)]. Akt is naturally maintained in an inactivated state. Interaction with 3-phosphoinositides induces a conformational change and recruitment to the membrane where Akt is activated (Chan and Tsichlis, 2001). Phosphorylation of Threonine-308 activates Akt, although phosphorylation at Serine-473 (Ser473) is required for maximal kinase activity (Alessi et al., 1996; Bellacosa et al., 1998). Following phosphate dependent activation, Akt dissociates from the membrane and goes on to activate downstream signaling cascades. Inactivation of Akt is mediated by dephosphorylation of Thr308 and Ser473 (Hers et al., 2011).

1.13.2 Akt Signaling

Akt is key regulator of cell survival, metabolism, protein synthesis, growth, and cell cycle regulation (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991; Datta et al.,

1999; Kandel and Hay, 1999; Vanhaesebroeck and Alessi, 2000). Akt signaling is mediated by serine/threonine phosphorylation of a wide range of downstream targets. Inhibition of apoptosis is achieved via inhibitory phosphorylation of pro-apoptotic factors such as GSK-3β (Cross et al., 1995; Srivastava and Pandey, 1998), Bcl-2-associated death promoter (BAD) (Datta et al., 1997; del Peso et al., 1997), and caspase-9 (Franke and Cantley, 1997; Song et al., 2008). The various downstream substrates of Akt have recently been reviewed (Hers et al., 2011). The current study focuses on the relationship between Akt and downstream factor GSK-3β (Section 1.12). Akt is the most well-defined upstream kinase of GSK-3β (Cross et al., 1995). Phosphorylation by Akt at Ser9 inactivates GSK-3β, and is proposed to underlie much of the cell survival effects attributed to Akt (Hers et al., 2011).

1.13.3 Akt after TBI

Due to the central importance of Akt in numerous signaling mechanisms, dysregulation of the kinase is linked to a variety of diseases such as cancer, diabetes, cardiovascular disease and some neurological disease. Akt has since been investigated following TBI considering its roll in cell survival and inhibition of pro-apoptotic factors. Using an *in vitro* TBI model, Neary and colleagues (2005) reported that mechanical strain increases Ser473 phosphorylation in astrocytes. This activation of Akt was proposed to be a neuroprotective mechanism in response to trauma (Neary et al., 2005). *In vivo* models have further supported the notion of increased Akt phosphorylation following brain injury (Noshita et al., 2002; Rubovitch et al., 2010; Park et al., 2012; Zhang et al., 2014; Du et al., 2016; Wang et al., 2017).

1.14 Summary and Objectives

TBI is the leading cause of death and disability in individuals under the age of 45 (Ghajar, 2000; Cole, 2004). mTBI, or concussion, is the most common form of head injury that

has been considered something of a ‘silent epidemic’ in modern times (Tellier et al., 1999). mTBI accounts for nearly 80% off all TBI (Bernstein, 1999; Anon, 2009), and produces a constellation of debilitating symptoms including, but not limited to, headaches, dizziness, confusion, and nausea, as well as learning and memory deficits (Baddeley et al., 1987; Dikmen et al., 1987; Ylvisaker and Feeney, 1998). Following injury, the brain is thought to be in a state of vulnerability in which a second injury could exacerbate pathophysiology (Hovda et al., 1991; Yoshino et al., 1991b). Those that have sustained a previous injury are at greater risk for sustaining additional head injuries, termed rmTBI (Barkhoudarian et al., 2011). Recovery from a mTBI is prolonged if a prior injury has occurred, and rmTBI may result in cumulative damage (Salcido and Costich, 1992; Elson and Ward, 1994; Kelly and Rosenberg, 1998). The juvenile population is of particular interest as children are known to have greater susceptibility to head injury (Centers for Disease Control and Prevention, 2000; Rutland-Brown et al., 2006).

The aim of the present work was to contribute to the characterization of the novel ACHI model. A relevant model of mTBI should produce a functional disturbance in the absence of gross pathology, as well as rapid presentation of symptoms that are short lived and resolve spontaneously. Initial investigations sought to identify the acute effect of rmTBI in fully conscious juvenile rodents. Behavioural tests were performed to determine whether the observed neurological impairment translates to neurobehavioural alterations similar to those seen in the clinical population. Additionally, biochemical analysis were utilized to determine if rmTBI produces tau hyper-phosphorylation.

Objective 1: To determine if the ACHI model produces acute neurological impairments.

Objective 2: To investigate if rmTBI induces neurobehavioural sequelae.

Objective 3: To examine tau protein phosphorylation following rmTBI.

2. General Methods

2.1 Animals

Long Evans rats were purchased (Charles River Laboratories, St. Constant, PQ) or bred at the University of Victoria. Pups were weighed and weaned at PND 21 and housed in same-sex groups of 2-3. Animals were housed in standard cages in a temperature (22.5˚C ± 2.5˚C) and light controlled (12-hour light/dark cycle) room with *ad libitum* access to standard food and water. Littermates were randomly assigned to rmTBI or sham experimental groups. At juvenile age (PND 25-28), male rats were subjected to rmTBI or sham procedures. All procedures were carried out in accordance with protocols approved by the Animal Care Committee at the University of Victoria and standards set by the Canadian Council for Animal Care.

2.2 Awake Closed Head Injury

Unanaesthetized juvenile rats were subjected to a novel model of mTBI, the ACHI model, which is based on a previously developed procedure for mice (Petraglia et al., 2014a) with modifications (**Figure 3**). Animals were inserted head-first into in a clear plastic restraint bag (Model DC-200, Braintree Scientific, Braintree, MA) and immobilized using a plastic clip placed behind the rat. The cone-shaped bag has a small opening to provide ventilation. A 3D printed (Replicator-2, MakerBot, Brooklyn, NY) helmet was secured to the animal's head using an elastic band and double-sided tape (**Figure 3**). The posterior edge of the helmet is positioned roughly 1mm rostral to the interaural line, and centered with the impact site over the left parietal cortex. The helmet functions to accurately deliver the injury and diffuse the force of impact across the surface of the skull. Rats were positioned on a three inch thick foam (Super-Cushioning Polyurethane Foam Sheet, McMaster-Carr, OH) platform below the injury device (**Figure 3)**. Placement on the foam platform allows for the acceleration-deceleration forces of injury.

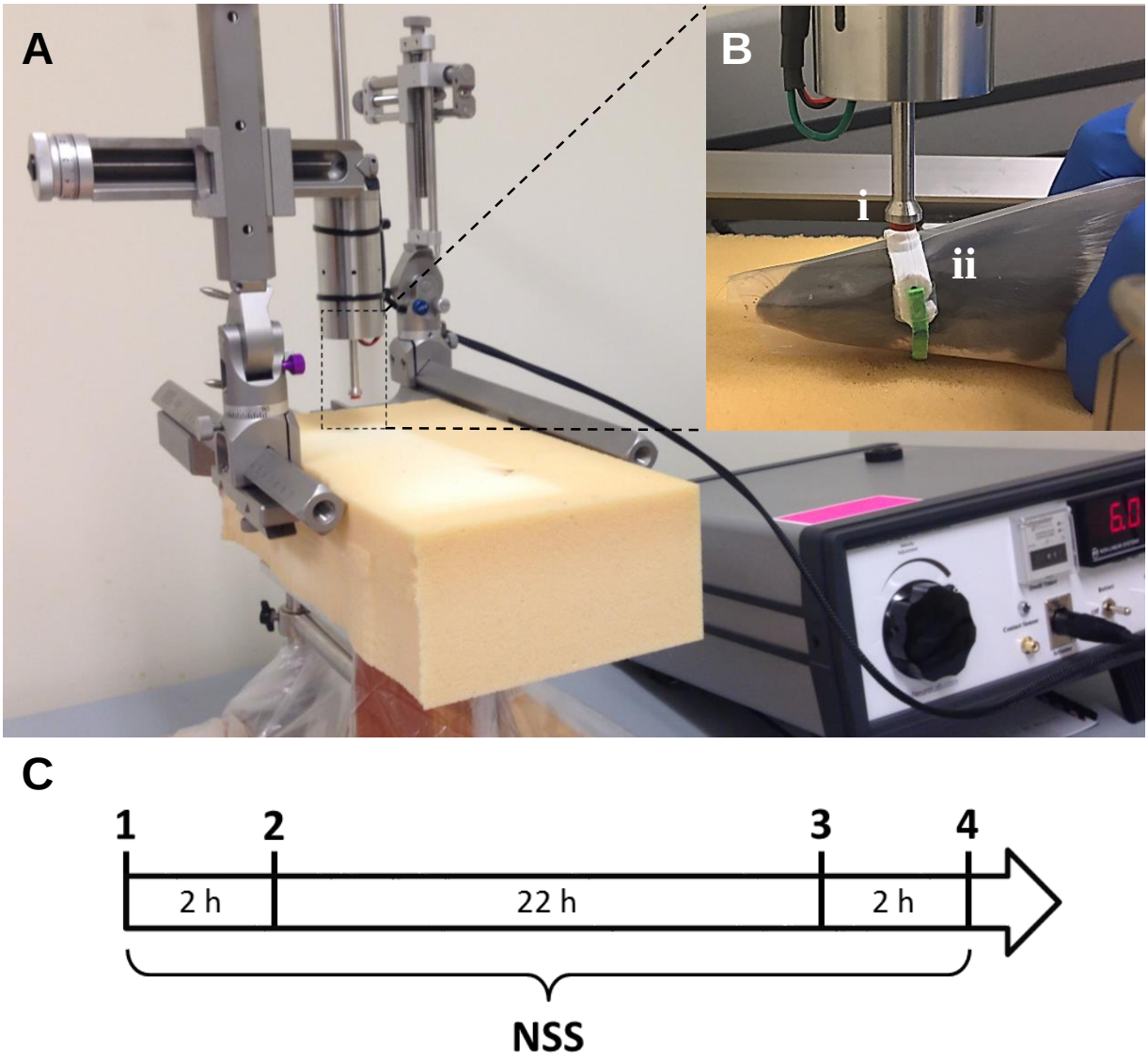

Figure 3. Awake Closed Head Injury apparatus. Image of the ACHI model and injury timeline. (A) Device overview: a modified Leica Impact One controlled impactor was used to deliver rmTBI to unanaesthetized juvenile rats. Impact velocity (6 m/s) and dwell time (10 ms) are set using the control unit. The stereotaxic frame holds the electromagnetic piston and can be adjusted to target impact. Restrained animals are placed over a soft foam platform. (B) Zoomed frame of electromagnetic piston; i) impactor with rubberized tip; ii) animal in restraint bag with 3D printed helmet. (C) rmTBI animals sustained two injuries per day with a two hour inter-injury interval. Sham animals underwent four sham procedures following the same timeline without impact. The Neurological severity score (NSS) was performed prior to the first injury and immediately after each impact or sham procedure.

A modified CCI device (Impact One, Leica Biosystems Inc, ON, Canada) was used to provide the repeat impacts. The device was vertically mounted on a stereotaxic frame and

modified with a 7mm rubberized tip (**Figure 3**). To perform an impact, the impactor tip was carefully lowered until it touched the surface of the helmet impact site (zero point). The tip was retracted, and the impactor positioned such that the tip is driven 10 mm beyond the zero point. Impacts are delivered at a velocity of 6 m/s with a duration of 10 ms (dwell time). Rats in the rmTBI group sustained a total of four impacts over a period of two days: two impacts per day, each separated by a 2 h period. Sham animals followed the identical protocol with the omission of the impacts. Immediately following each impact or sham procedure, animals were removed from the restraint bag and subjected to neurological assessment (see Section 3.2).

2.3 General statistical analysis

Statistical analyses were performed using Statistica 7.0 analytical software (Statsoft Inc., Tulsa, OK, USA) and RStudio (RStudio, Boston, MA). All data are presented as the Mean ± SEM. Behavioural data was analyzed with the Friedman's test, Chi-square test, Wilcoxon rank-sum test or two-tailed Student's *t*-test as appropriate. Western blotting data was analyzed with two-tailed Student's *t*-tests. A p value of < 0.05 was considered to be statistically significant. Details on the specific statistical analyses used for individual experiments are outlined in greater detail in corresponding Methods sections of the following chapters.

3. Evaluation of neurobehavioural sequalae following rmTBI

This Chapter is based in part on the following manuscript:

Alicia Meconi, **Ryan C. Wortman**, David K. Wright, Katie J. Neale, Sandy R. Shultz, and Brian R. Christie. A model for repeated closed-head injury in non-anaesthetized juvenile rats. *Journal of Neuroscience Methods.* (Submitted).

3.1 Background

3.1.1 Neurological Severity Score

A major benefit of the ACHI model is the lack of anaesthesia, which allows the researcher to make an immediate neurological assessment following impact, without the delay or confounds associated with anaesthesia or surgery. The neurological severity score (NSS) is used to assess basic neurological outcomes following each injury or sham procedure. The NSS test used herein is based on prior research (Shapira et al., 1988; Shohami et al., 1995; Schaar et al., 2010; Ding et al., 2013) and includes three assessments for level of consciousness, followed by four simple reflexive and motor tasks.

3.1.2 Open-Field test

The open-field test was developed by Hall (Hall, 1934), and is now the most popular test for measuring spontaneous activity. Anxiety-like behaviour in the open-field is based on two innate traits of rodents: (1) agoraphobia, or fear of open spaces; (2) individual isolation (Kessler et al., 1999). The relative amount of time the animal spends in the centre compared to the periphery is a measure of anxiety. Rodents are naturally exploratory, but also tend to remain near the peripheral regions of an environment, termed thigmotaxis (Crawley, 1985, 1999). Anxiolytic drugs decrease anxiety-like behaviours of rodents in the open-field (Stefański et al., 1993; Rex et al., 1998). Numerous studies have investigated the effect of TBI on anxiety-like behaviour in the open-field using mice (Kane et al., 2012; Washington et al., 2012; Mannix et al.,

2014; Mouzon et al., 2014; Petraglia et al., 2014a; Nichols et al., 2016) and rats (Jones et al., 2008a; Pandey et al., 2009; Kwon et al., 2011; Shultz et al., 2011; Almeida-Suhett et al., 2014; Ghadiri et al., 2014; Arain et al., 2015; Mychasiuk et al., 2015).

3.1.3 Elevated-Plus Maze

The EPM is a widely used tool for evaluating exploratory behaviour. Rodents have a tendency to explore novel environments, but display a natural preference for dark, enclosed spaces (Pellow et al., 1985). The closed arms of the EPM are walled and provide a hiding spot, while the open arms are brightly lit, exposed, and elevated, and therefore considered aversive. The EPM assesses this natural conflict between these two traits (Blumstein and Crawley, 1983). Compared to control animals, rodents exhibiting impulsivity or risk-taking behaviour will spend increased amounts of time exploring the open arms of the apparatus (Lindema et al., 2008; Meyer et al., 2008; Bortolato et al., 2009; Mosienko et al., 2012). The EPM has previously been employed to assess risk-taking behaviour following TBI (Shultz et al., 2011; Washington et al., 2012; Johnson et al., 2013; Logsdon et al., 2014; Turner et al., 2015).

3.1.4 Rota-Rod

Vestibulomotor tests are designed to evaluate fine motor coordination, and are beneficial for assessing functional recovery in experimental models of TBI as they translate to actions such as coordination, balance and walking in the clinical setting. One such behavioural tool is the Rota-Rod test, first described by Dunham and Miya (1957), and later adapted with accelerating protocols (Jones and Roberts, 1968). The task requires that the animal maintain balance and coordination on a rotating rod that gradually increases in speed of rotation over the course of the testing period. The use of a rod also takes into account grip strength, which declines post-TBI (Hall et al., 1988). The Rota-Rod was first implemented with TBI research in 1994 to compare

the utility of the Rota-Rod to the previously established beam-balance and beam-walking tests following brain injury (Dixon et al., 1987, 1991). All tests were evaluated to detect deficits in motor function after mTBI or moderate TBI. Only the Rota-Rod was sensitive enough to detect mTBI-induced motor dysfunction, and likely measures unique or additional aspects of motor impairment than beam-balance or beam-walk (Hamm et al., 1994).

3.1.5 Forced Swim Test

Porsolt and colleagues (1977) developed a rodent model of behavioural despair, which is defined as a significant decline in the animal's effort to escape an aversive situation. Animals are forced to swim in a container with no means of escape. Eventually, the subjects accept the experimental conditions and make only limited movements to maintain their head above water. This behavioural immobility reflects a state of despair where the animal has learned helplessness. Interestingly, the duration of immobility can be reduced by anti-depressant drugs. Increased immobility compared to control animals is considered depressive-like behaviour (Porsolt et al., 1977, 1978). Considering the prevalence of depression following TBI in humans, the Porsolt Forced Swim Test (FST) is commonly implemented in TBI research with mice (Washington et al., 2012; Petraglia et al., 2014a; Nichols et al., 2016) and rats (Jones et al., 2008a; Taylor et al., 2008; Shultz et al., 2011, 2015; Mychasiuk et al., 2015; Tan et al., 2016).

3.2 NSS procedure

The entirety of the NSS is completed within the first minute post-injury as possible. Absence of consciousness or reflexes were timed until recovery, and each other component of the test was scored in a pass/fail fashion. The NSS is performed by removing the animal from the restraint bag onto a clean surface immediately after impact or sham procedure. An initial assessment of (1) apnea, or absence of breathing, is made. If breathing is absent, the duration to

return is recorded. Next, the (2) toe pinch reflex (**Figure 4A**) is evaluated by extending the animal's contralateral (to injury hemisphere) hind limb and firmly pinching. Immediate flexion of the limb is a positive reflex. Failure to retract the limb is timed and the pinch is repeated at five second intervals until the reflex returns. Righting reflex (3) is evaluated by placing the animal in the supine position and recording the time to pronate (**Figure 4B**). Immediate (<1s) return to prone position is indicative of an intact reflex. Absence of righting reflex is timed until return. These three initial assessments of level of consciousness must first be completed prior to proceeding to the remaining four simple reflexive and motor tasks of the NSS.

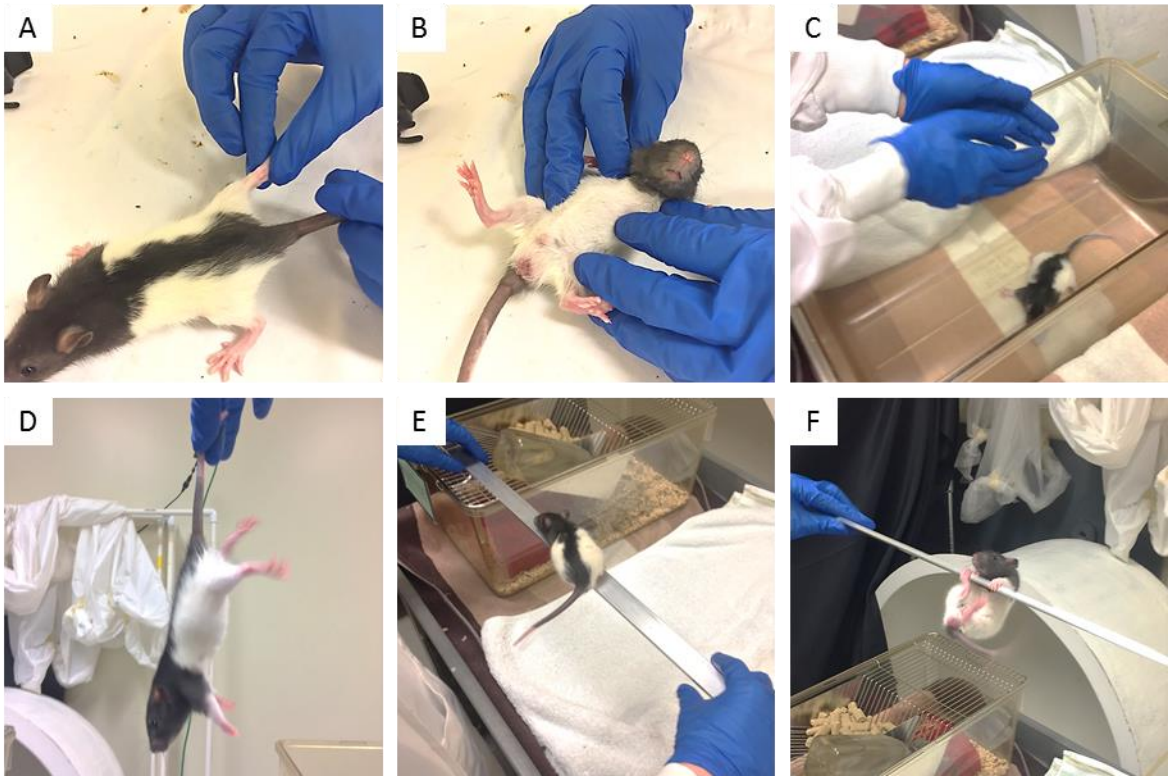

Figure 4. Neurological severity score. Representative images of NSS procedures in order of testing. The NSS is performed after each injury and begins with assessment of apnea, followed by toe pinch reflex (A), Righting reflex (B), Startle response (C), Limb Extension (D), Beam Walk (E), and Rotating Beam (F).

To examine the (4) startle response (**Figure 4C**), the animal is placed in an empty, standard housing cage, and an intense acoustic stimulus (hand clap) is presented directly above

the cage. A pass is scored for a stereotyped reflexive flinch in response to the acoustic stimulus. The animal is then grasped by the base of the tail and raised approximately 60 cm into the air to test the (5) limb extension (**Figure 4D**) response. Full extension of both forelimbs constitutes a pass. The animal is then immediately placed on flat, narrow balance beam to examine their ability to (6) balance and walk across (**Figure 4E**). The beam, 100 cm long x 2 cm wide x 0.75 cm thick, extends from an empty cage to the animal's home cage and is raised 22 cm above a padded work surface. Animals are placed squarely balanced at the center of the beam facing their home cage. A pass is scored if the animals traverse the beam within 10 seconds of placement. Immobility, inability to grasp the beam with each limb, or falling within the 10 second period constitutes a fail. Lastly, the animal's dexterity is evaluated on a slowly (7) rotating beam (**Figure 4F**). The animal is again placed squarely balanced at the center of the aforementioned beam, and then elevated to a height of 75 cm above a padded work surface. The beam is rotated at a speed of 1 rotation per second for a total of four rotations. Successfully remaining on the beam for the duration of the rotations is scored as a pass, and falling off a failure. Equipment used for the NSS is thoroughly cleaned between animals with 70% ethanol.

Each animal completed NSS testing a total of five times. Baseline values were collected prior to the first injury, and NSS testing was performed immediately after each injury or sham procedure. Apnea, toe pinch, and righting reflex were recorded if present. Startle, Limb Extension, Beam Walk, and Rotating Beam were each scored as pass/fail where successful completion of the task was awarded a point for a maximal performance score of 4 (**Table 2**).

Table 2. Neurological Severity Score parameters.

Task	*Description*	*Score*[a]
Apnea	Assess breathing	Time (s)
Toe pinch	Innate reflex assessment	Time (s)
Righting reflex	Innate reflex assessment	Time (s)
Startle	Innate reflex assessment in response to acoustic stimulus	0/1
Limb Extension	Respond with full extension of fore limbs	0/1
Beam Walk	Ability to cross a 100 cm long x 2 cm wide beam	0/1
Rotating Beam	Ability to hold onto beam when rotated at 1 rotation/s	0/1
Maximal score		4

[a] One point is awarded for successful completion of task

3.3 Behavioural testing

All behavioural testing was conducted during the light phase of the cycle, and in a separate, dedicated behaviour room within the animal care unit. Rats were acclimated to the room during ACHI procedures. The behavioural experiments were designed to test for co-morbid neurobehavioural and motor deficits, such that the same rat was used for multiple behavioural tests. Open-field, EPM, and Rota-Rod testing was performed, in that order, on PID 1. Each animal had a minimum one hour break between tasks. Animals not euthanized on PID 1 performed a second testing session on the Rota-Rod on PID 7. An exception was the FST, which is designed to cause despair, such that separate cohorts were designated only to this behavioural test. An additional cohort was tested in only the open-field on PID 7 to ensure spontaneous behaviour in the novel arena. A timeline of experimental procedures is presented in **Figure 5**.

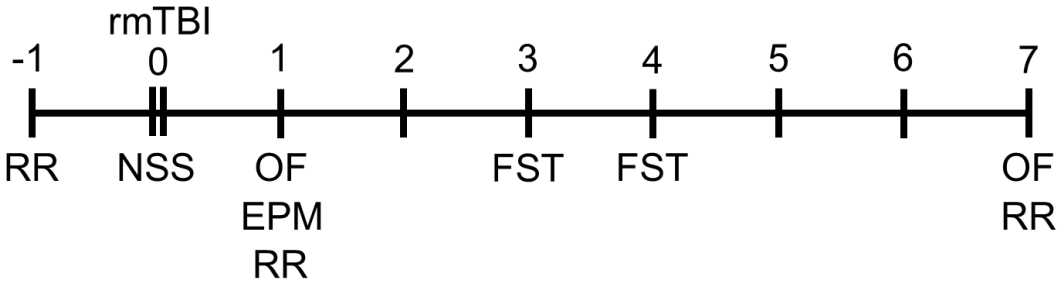

Figure 5. Timeline of experimental procedures. Outline displays timing of all behavioural tests and experimental procedures, beginning 24 h prior (Day -1) to ACHI procedures (Day 0). (RR, rota-rod; NSS, neurological severity score; rmTBI, traumatic brain injury; OF, open-field test; EPM, elevated-plus maze; FST, forced swim test).

3.3.1 Open-Field protocol

Gross locomotor ability and anxiety-like behaviour was assessed on PID 1 and PID 7 (separate cohorts) using the open-field test as previously described (Jones et al., 2008a; Shultz et al., 2015; Tan et al., 2016). Animals were placed in the center of a white, novel arena (100 cm diameter) in a brightly lit room (4 x 500 W halogen lamps) and given 5 min to explore freely. Animals were tracked with EthoVision XT 11.5 software (Noldus, Netherlands). Increased time spent in the margins (thigmotaxis) or decreased time spent in the centre area (70 cm diameter) are measures of anxiety-like behaviour (Prut and Belzung, 2003; Jones et al., 2008b). Secondary measures included total horizontal distance traveled, and average velocity.

3.3.2 Elevated-Plus Maze protocol

On PID 1, after completion of the open-field test, risk-taking behaviour was assessed using the EPM. The EPM apparatus (Noldus) consists of a plus-shaped platform raised 62 cm above the ground. One set of opposing arms are fully exposed (open arms; 50 cm long x 10 wide cm) and the other opposing arms are enclosed with walls (closed arms; 50 cm long x 10 cm wide x 30 cm tall). Rats were placed at the centre junction of the maze facing an open arm and allowed to explore freely for 5 mins. The number of entries into each arm and total time spent in each arm was recorded by overhead camera and analyzed using EthoVision XT 11.5 software

(Noldus). The software tracked the centre point of the rat body, and an entry was scored when the centre point crossed into the arm.

3.3.3 Rota-Rod protocol

Motor coordination and balance were assessed on PID 1 and PID 7 using the Rota-Rod (Rat Rotarod NG, Model 47750; Ugo Basile, Varese, Italy). The apparatus consists of a 6 cm diameter rotating rod with machined grips, divided into four equal 8.7 cm wide sections raised 30 cm above trip boxes. An accelerating protocol was used with the speed of rotation increased from 10 – 50 rpm over 300s. Three test trials were conducted with a minimum of 5 min rest in home cages between trials. Each trial was terminated if an animal fell, turned around within the initial 30 s of the trial, clung and rotated for two full rotations, or remained on for >300s. Latency to fall (s), maximum speed (rpm), and distance traveled (m) were automatically recorded for each trial. The average of the three trials was calculated for each measure and used for analysis. Training trials and baseline values were recorded 24 h prior to ACHI procedure.

3.3.4 Forced Swim test protocol

The FST was conducted on a designated cohort of animals on PID 3-4 to evaluate depression-like behaviour (Porsolt et al., 1977). A clear glass cylinder (23.5 cm diameter, 40.5 cm high) was filled with water (25°C ±2) to a depth of 30 cm. On PID 3, rats completed a 15 min forced swim training session in the swim apparatus. Rats were then subjected to a 5 min test session in the swim apparatus on PID 4 (Porsolt et al., 1977; Shultz et al., 2011, 2012a, 2012b). Behaviour was recorded by side-view camera and level of activity analysed by an individual blind to experimental group using EthoVision XT 11.5 software (Noldus). Activity thresholds (low, medium, high) were set based on activity in the swim apparatus: low, defined as minimum movements required to maintain head above water; medium, defined as active swimming; and

high, defined as the rat actively struggling to escape the cylinder. Only those behaviours that persisted for greater than 2 s were scored. Increased immobility (low threshold activity) duration represents depressive-like behaviour (Porsolt et al., 1977).

3.4 Statistical analysis

rmTBI and sham animals each performed the NSS a total of five times including baseline and after each injury or sham procedure. Based on the scoring criteria of the NSS and ordinal data, non-parametric statistical tests were utilized. The Wilcoxon rank-sum test was used to determine the difference between average sham and rmTBI NSS scores within each injury trial. Group Effect size was also calculated for each injury trial of the NSS. The Friedman's Test was used to compare NSS performance in each group across injuries. *Post hoc* analysis was performed with the Conover-Iman test of multiple comparisons using rank sums. The Bonferroni correction was applied for *p*-value adjustment. Pearson's Chi-squared test with Yates' continuity correction was used for comparing sham and rmTBI NSS scores on individual tasks (Startle, Limb Extension, Beam Walk, and Rotating Beam). The Open-field test, EPM, Rota-Rod, and FST were analyzed using the two-tailed Student's *t*-test. A p-value of < 0.05 was considered to be statistically significant.

3.5 Results

3.5.1 rmTBI alters consciousness acutely post-injury

Conscious state was determined immediately after each ACHI or sham procedure by evaluation of the initial three components of the NSS, namely presence of apnea, loss of toe pinch reflex, and loss of righting reflex. No sham animals displayed altered consciousness following restraint, such that only rmTBI animal data is reported in **Table 3**. Apnea was not detected following any injury. The toe pinch reflex was lost on six occasions in a total of five animals (28.8%). The mean duration of loss of toe pinch reflex was 4.6 seconds. The righting

reflex was absent in 47.8% of injured animals. Latency to recover righting reflex ranged from 1-134 seconds (data not shown) with an average duration of 28.8 seconds. These NSS findings are summarized in **Table 3.** Concussive convulsions (CC) were also observed in response to injury, occurring in 12 animals or 52.2%. A total of 17 CC events were witnessed with an average duration of 16.6 seconds. A mortality rate of 4.3% was determined (**Table 3**).

Table 3. Frequency of acute changes to consciousness following rmTBI.

	Apnea	Toe pinch reflex	Righting reflex	CC	Mortality
Frequency (n/total n)	0/23	5/23	11/23	12/23	1/23
Percent (%)	0	21.7	47.8	52.2	4.3
Total events (#)	0	6	15	17	1
Average duration (s)	0	4.6	28.8	16.6	n/a

CC, Concussive convulsions

3.5.2 rmTBI impairs simple reflexes and gross motor performance

NSS testing was performed immediately after each injury or sham procedure to assess basic neurological outcomes. The composite NSS score for each group was determined for each of the five impacts (**Figure 6**). Sham and rmTBI animals were equally proficient at baseline ($W = 192$, $p = 0.55$, $r = -0.094$). Average NSS scores differed significantly between sham and rmTBI animals after the first ($W = 350$, $p < 0.001$, $r = -0.64$), second ($W = 384.5$, $p < 0.001$, $r = -0.77$), third ($W = 398.5$, $p < 0.001$, $r = -0.81$), and fourth ($W = 398.5$, $p < 0.001$, $r = -0.81$) impacts. Sham animals displayed no significant difference in NSS performance over the 5 trials ($\chi^2(4) = 3$, $p = 0.5578$), whereas rmTBI animal NSS scores were significantly different across trials ($\chi^2(4) = 43.11$, $p < 0.001$). *Post-hoc* analysis with Bonferroni correction revealed that NSS performance significantly decreased following each injury compared to baseline ($p < 0.001$ for

each impact). There was a significant decrease in score between the first injury and all subsequent injuries ($p < 0.001$ for each additional injury compared to the first).

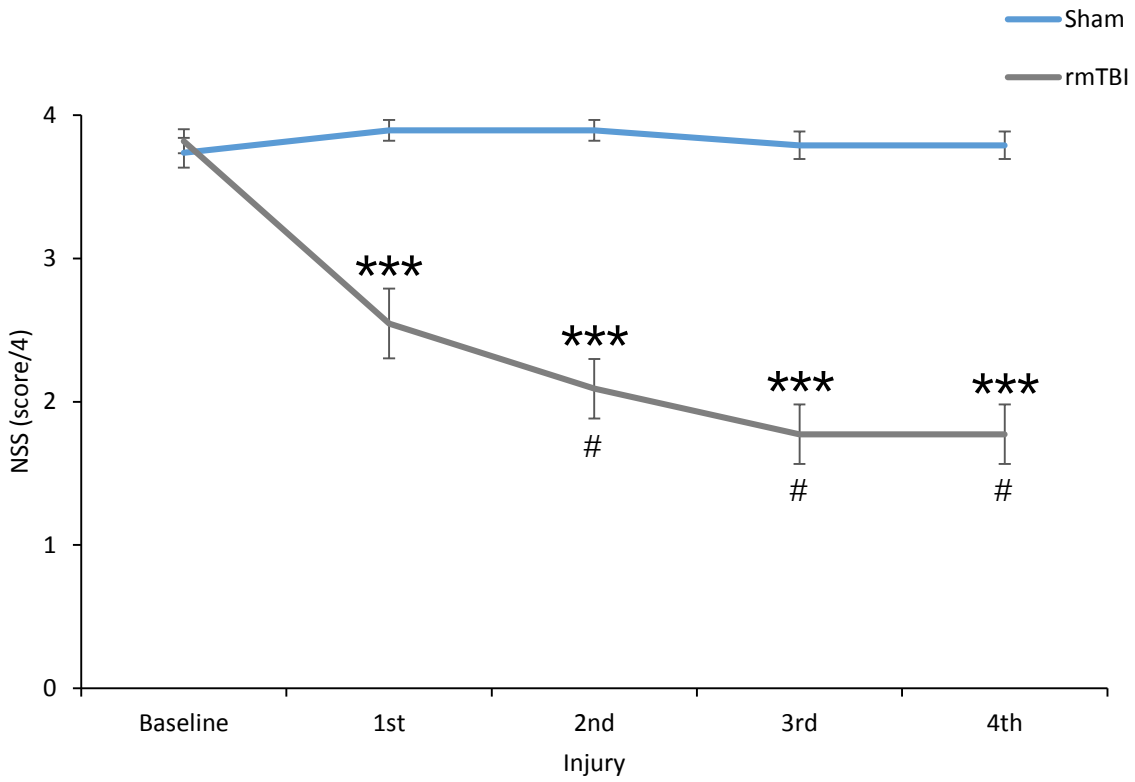

Figure 6. rmTBI causes acute neurological impairment in juvenile rats. Neurological severity score (NSS) performance was significantly decreased following each injury in rmTBI animals compared to sham controls. Sham scores did not differ across trials (***$p < 0.001$). NSS scores from the 2nd, 3rd, and 4th injuries were significantly reduced compared to the initial injury (# $p < 0.001$). Data presented as Mean ± SEM. Sham: n = 19; rmTBI: n = 22.

Performance on each task of the NSS was evaluated individually (**Figure 7**). The Startle response task did not differentiate between sham and rmTBI animals with no difference in performance ($\chi^2(1) = 0.37$, $p = 0.54$). rmTBI animals showed significantly reduced scores on the Limb Extension ($\chi^2(1) = 59.47$, $p < 0.001$), Beam Walk ($\chi^2(1) = 97.17$, $p < 0.001$), and Rotating Beam ($\chi^2(1) = 30.34$, $p < 0.001$) tasks compared to sham.

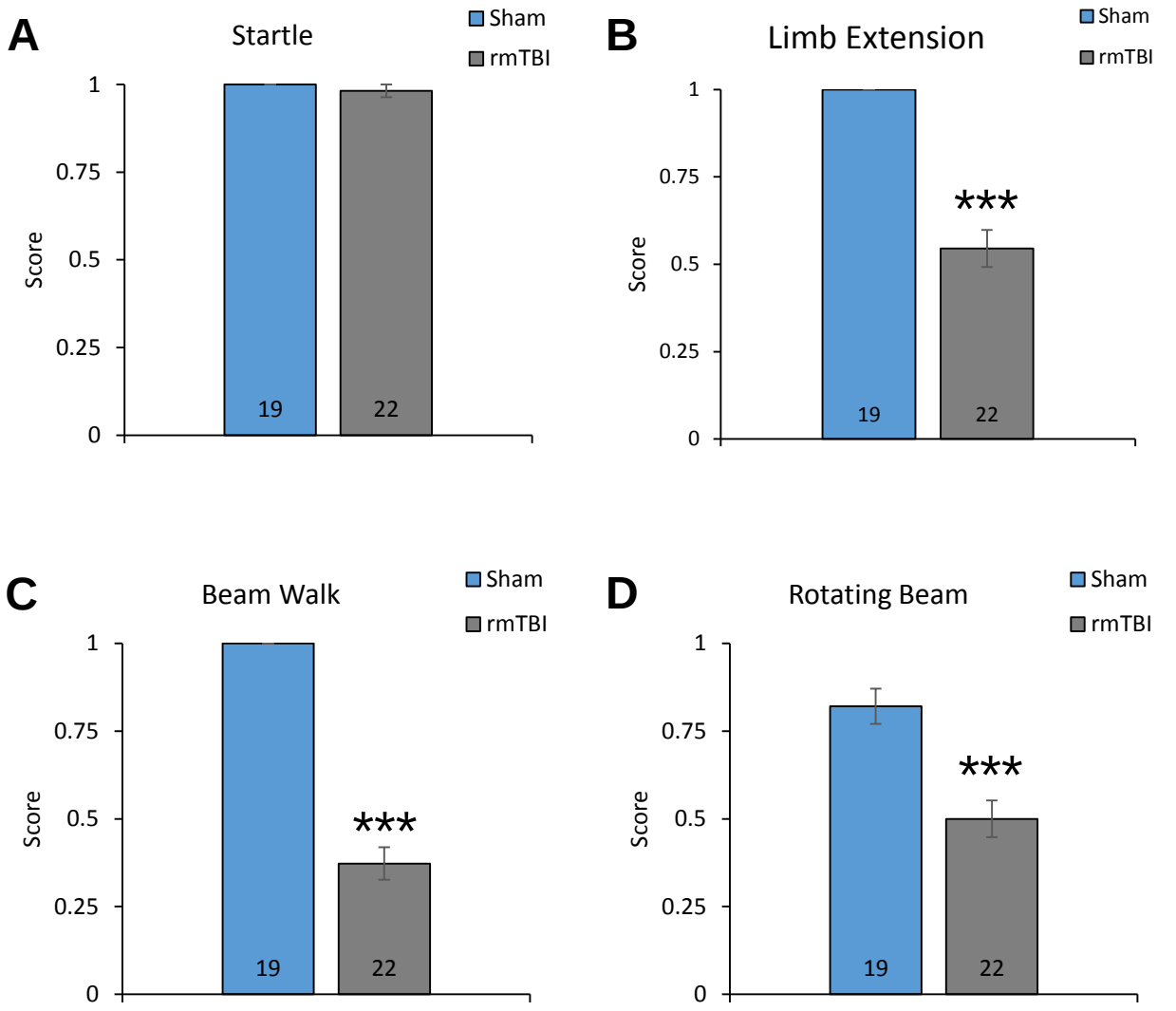

Figure 7. rmTBI animals display deficits on specific NSS tasks. (A) Startle response was not significantly different between groups. (B) Limb Extension, (C) Beam Walk, and (D) Rotating Beam were all efficient tasks for differentiating between groups. rmTBI animals performance on each of these tasks were significantly impaired compared to sham animals. Data presented as Mean ± SEM. ***$p < 0.001$.

3.5.3 rmTBI induces anxiety-like behaviour 24 hours post-injury

On PID 1, rats were subjected to open-field testing and their behaviour recorded over a 5 min period. rmTBI animals travelled significantly less distance in the centre ($t_{(16)} = 2.67$, $p = 0.017$; **Figure 8A**). Anxiety-like behaviour was detected in rmTBI animals as significantly increased time in the perimeter ($t_{(16)} = -2.81$, $p = 0.012$; **Figure 8B**) and significantly decreased time in the centre of the open-field ($t_{(16)} = 2.82$, $p = 0.012$; **Figure 8B**) compared to sham animals. There was no difference in total horizontal distance travelled ($t_{(16)} = 1.35$, $p = 0.196$; **Figure 8C**) or speed of travel ($t_{(16)} = 1.34$, $p = 0.199$; **Figure 8D**) suggesting that the injury did not cause motor impairment. Representative heat maps generated by the tracking software show that rats of both groups spent the majority of time in the perimeter of the maze, but sham controls spent a larger portion of time in the centre of the field (**Figure 8E**).

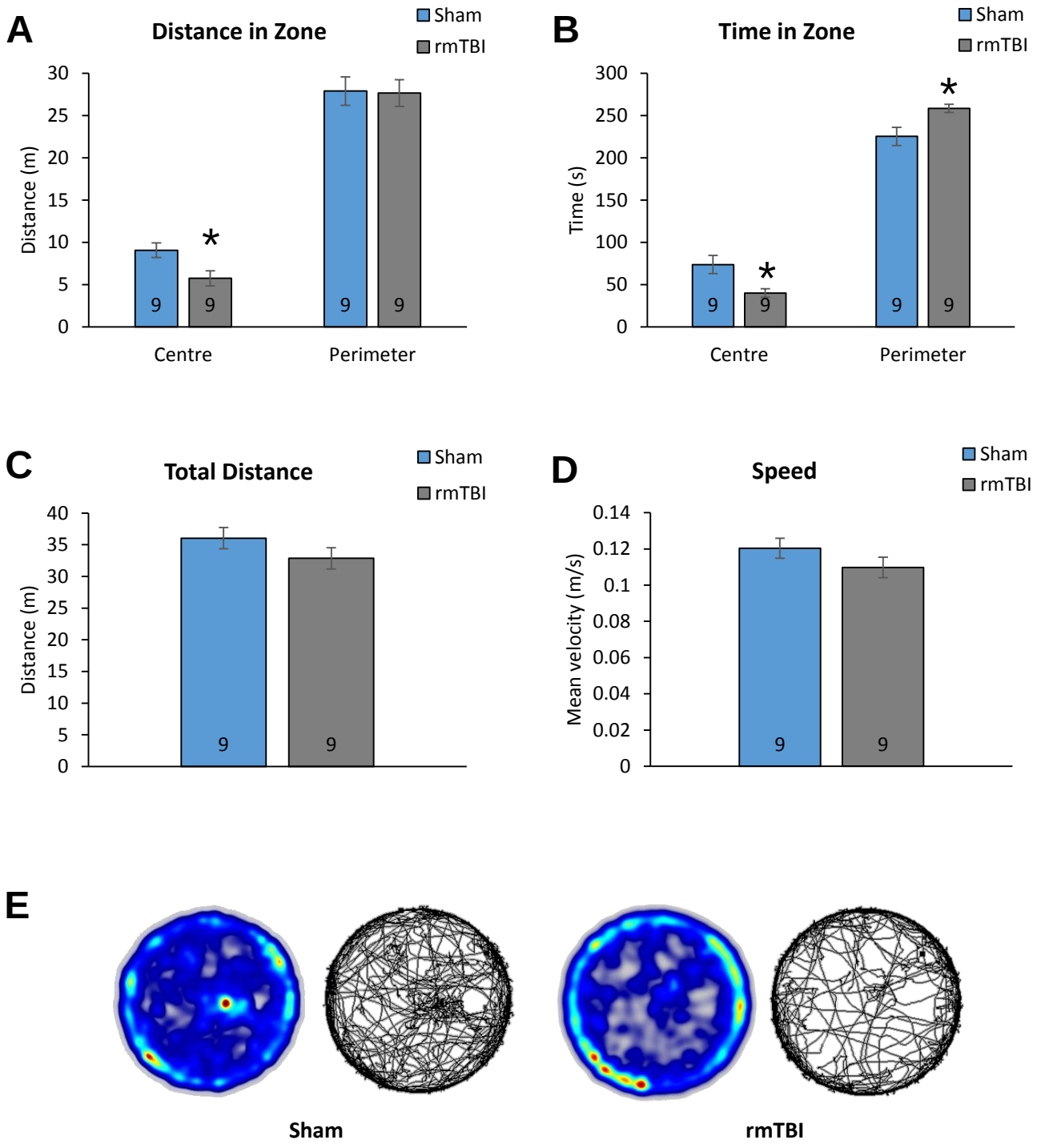

Figure 8. rmTBI injury induces anxiety-like behaviour in the open-field on PID 1. rmTBI rats display significantly decreased distance travelled in the centre (A), decreased time spent in the centre of the open-field and increased time in the perimeter (B) compared to sham animals. No differences are observed in total distance travelled (C) or speed of travel (D) in the field. (E) Representative tracking maps generated with EthoVision software display search path and strategies. Data presented as Mean ± SEM. $*p < 0.05$.

In a separate cohort of animals, no significant differences between rmTBI and sham animals were observed when tested on PID 7 in the open-field. Both groups travelled the same distance in the centre ($t_{(8)} = 0.777$, $p = 0.460$; **Figure 9A**). No anxiety-like behaviour was noted on PID 7 as measured by time in the perimeter ($t_{(8)} = -0.256$, $p = 0.804$; **Figure 9B**) or time spent in the centre of the open-field ($t_{(8)} = 0.350$, $p = 0.735$; **Figure 9B**). No differences were detected in total distance travelled ($t_{(8)} = 0.986$, $p = 0.353$; **Figure 9C**) or speed of travel in the maze ($t_{(8)} = 0.950$, $p = 0.370$; **Figure 9D**). Representative heat maps generated by the tracking software show that rats of both groups spent the majority of time in the perimeter of the maze, but sham controls spent a larger portion of time in the centre of the field (**Figure 9E**).

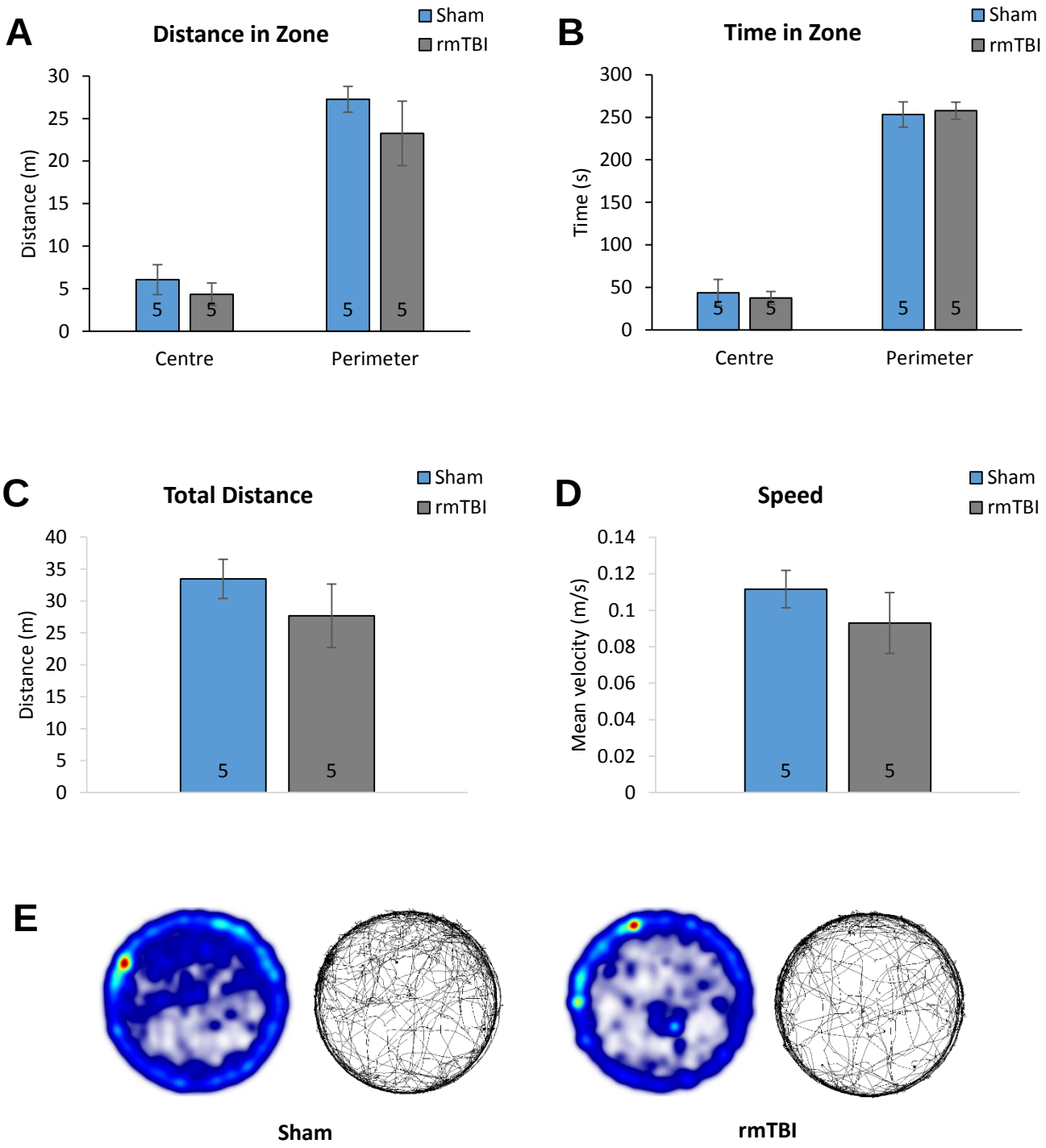

Figure 9. Anxiety-like behaviour caused by rmTBI diminished by PID 7. A separate cohort of animals tested in the open-field on PID 7 exhibited no anxiety-like behaviour after rmTBI. Injured animals travelled the same distance by zone (A) and spent similar time in either zone (B) as sham controls. No differences were observed in total distance travelled (C) or speed of travel (D) in the field. (E) Representative tracking maps generated with EthoVision software display search path and strategies. Data presented as Mean ± SEM.

3.5.4 rmTBI does not produce risk-taking behaviour

The EPM was performed to determine the effects of rmTBI on risk-taking behaviour. rmTBI animals did not display significantly different exploration in the EPM compared to sham controls 24 hours post-injury (**Figure 10**). The number of open arm entries ($t_{(15)} = 0.32$, $p = 0.75$) and total time spent in the open arms ($t_{(15)} = 0.43$, $p = 0.67$) were not significantly different than that of shams. No difference between groups was observed in closed arm activity (data not shown). This data indicates that rmTBI did not alter risk-taking behaviour.

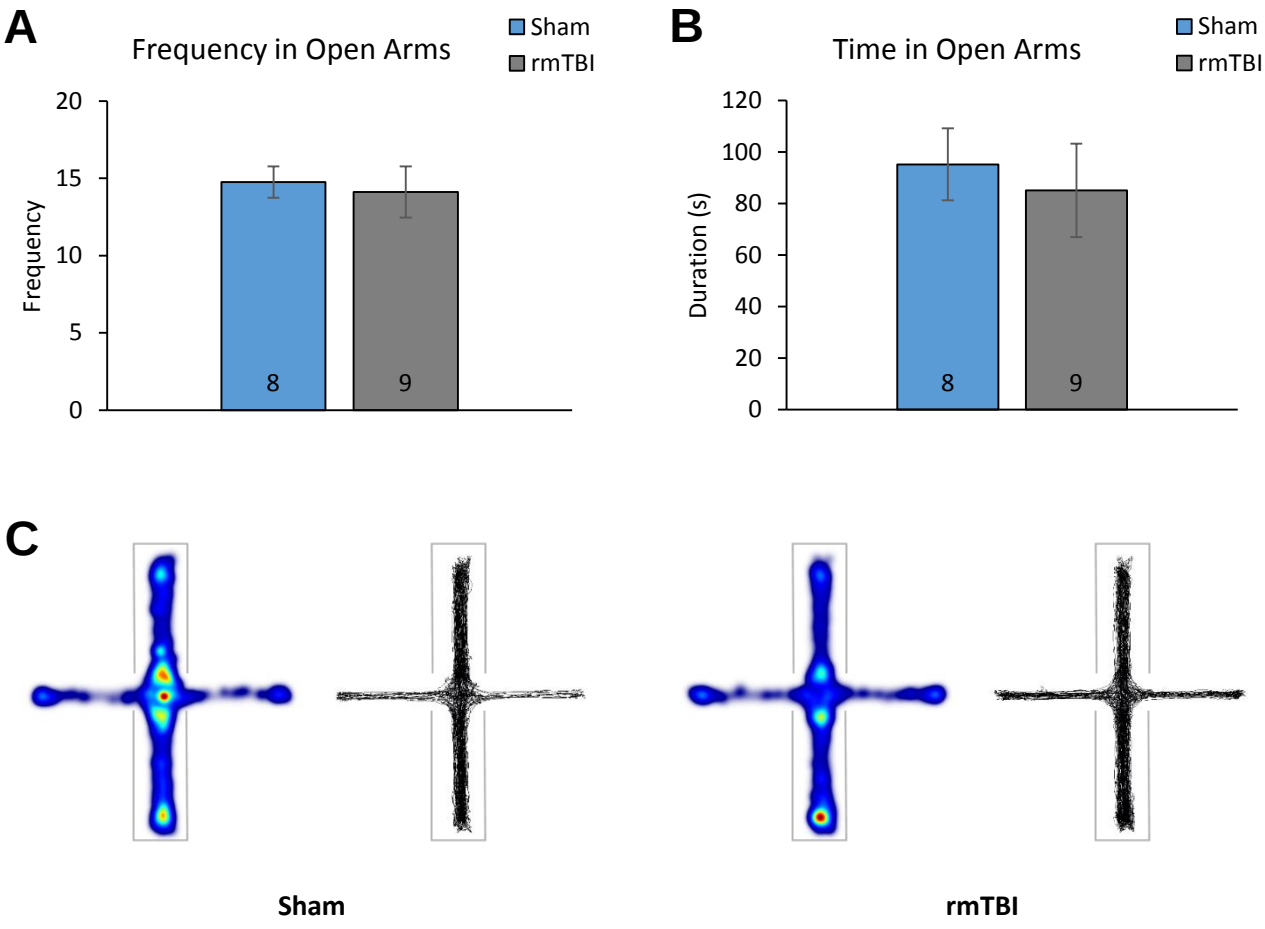

Figure 10. Activity in the Elevated-Plus Maze is not altered by rmTBI on PID 1. rmTBI animals do not exhibit risk-taking behaviour on PID 1 as measured by the number of entries into open arms (A) and total time spent in the open arms (B). (C) Representative tracking maps generated with EthoVision software display search path and strategies. Data presented as Mean ± SEM.

3.5.5 rmTBI decreases sensorimotor function

Animals were evaluated for motor deficits using the Rota-Rod test. Baseline values were recorded one day prior to injury or sham procedure and showed no differences between groups in all measures: latency ($t_{(16)} = 1.32$, $p = 0.204$), max speed achieved ($t_{(16)} = 1.09$, $p = 0.292$), and cumulative distance traveled ($t_{(16)} = 1.42$, $p = 0.174$). Following rmTBI, injured animals exhibited poorer performance in latency ($t_{(15)} = 2.63$, $p = 0.019$), max speed achieved ($t_{(15)} = 2.42$, $p = 0.029$), and distance traveled ($t_{(15)} = 3.08$, $p = 0.008$) compared to sham on PID 1. The deficits identified at 24 hour post-injury subsided by PID 7, where no significant differences were observed in latency ($t_{(8)} = 0.684$, $p = 0.514$), max speed achieved ($t_{(8)} = 0.792$, $p = 0.451$), and distance traveled ($t_{(8)} = 0.880$, $p = 0.404$) between rmTBI and sham animals. Combined Rota-Rod data is presented in **Figure 11.**

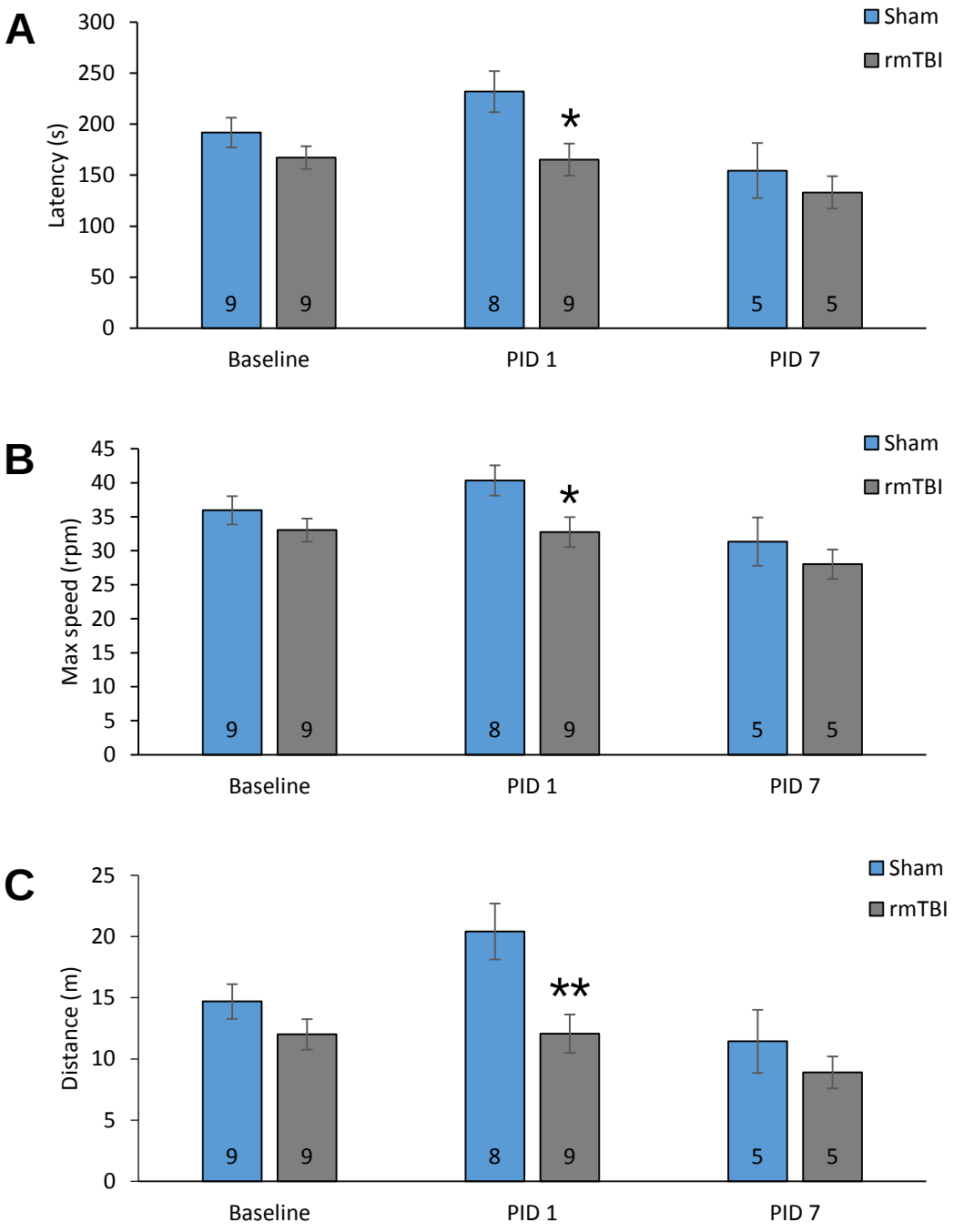

Figure 11. Effect of rmTBI on Rota-Rod performance. Motor coordination and balance was assessed prior to injury (baseline), on PID 1 and PID 7 using the Rota-Rod. Injured animals displayed significantly reduced performance on all measures compared to sham at PID 1. (A) Latency, (B) maximum speed achieved, and (C) distance travelled. Deficits were no longer present by PID 7. Data presented as Mean ± SEM. *$p < 0.05$; **$p < 0.01$.

3.5.6 rmTBI does not result in depressive-like behaviour

rmTBI rats did not exhibit depression-like symptoms on PID 4 as measured by low threshold activity in the FST (**Figure 12**). No differences were observed in low ($t_{(8)}$ = 0.21, p = 0.84), medium ($t_{(8)}$ = 0.11, p = 0.92), or high ($t_{(8)}$ = -0.19, p = 0.86) activity thresholds between rmTBI or sham control groups.

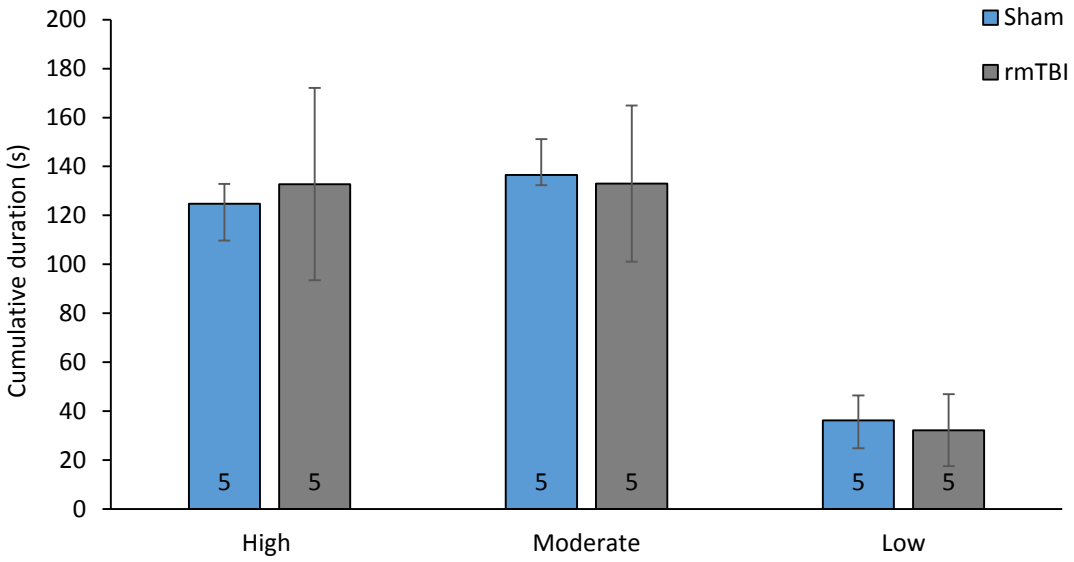

Figure 12. No depressive-like response to rmTBI on PID 4. Behavioural despair was evaluated on PID 4 using the FST. Injured animals did not display different amounts of time exhibiting low threshold activity compared to shams. No differences were observed in any activity threshold durations. Data presented as Mean ± SEM.

3.6 Discussion

A major benefit of the ACHI model is the lack of anaesthesia, which allows the researcher to make an immediate neurological assessment following each impact. The NSS test used herein is based on prior research (Shapira et al., 1988; Shohami et al., 1995; Schaar et al., 2010; Ding et al., 2013) and includes three assessments for level of consciousness, followed by

four simple reflexive and motor tasks. The NSS is, by design, a simple assessment of neurologic function that can be rapidly completed within the first minute after brain trauma. Consequently, it does not address more complex cognitive, motor, and emotional changes associated with clinical mTBI (Gibb and Kolb, 1998; Carroll et al., 2004; McCrea et al., 2009; McCrory et al., 2013).

3.6.1 Altered consciousness following rmTBI

The findings presented herein show that the ACHI model acutely alters consciousness post-injury. The large majority of animals display one or more changes to state of consciousness immediately after injury as measured by the initial three components of the NSS: Apnea, Toe pinch reflex, and Righting reflex (**Table 3**). No control animals displayed changes to consciousness after sham procedure. Injured animals did not display apnea at baseline or after any of the four injuries. Toe pinch reflex was lost in five animals, or 21.7%, for an average duration of 4.6 seconds. Loss of righting reflex was observed in nearly half the animals at an average duration of 28.8 seconds. Convulsions are not a component of NSS used in the present study, but were observed on several occasions following injury with the ACHI model. Indeed, over half of the injured animals exhibited CC, which lasted for an average of 16.6 seconds (**Table 3**). Although uncommon, CC are also reported following sports-related head injuries. In humans, CC are defined as a non-epileptic phenomena that occur within two seconds of impact. The pathophysiology of CC requires further investigation, but is proposed that the impact causes transient decerebration that results in loss of cortical inhibition and consequently convulsive movements (McCrory and Berkovic, 1998). CC are not commonly reported in experimental literature, as anaesthesia inhibits immediate assessment of neurological outcomes. In contrast to the current study, Petraglia and colleagues (2014) do not observe seizure activity in their awake

mouse model of rmTBI (Petraglia et al., 2014b). The experimental CC identified herein represents a novel finding in rodents sustaining rmTBI without anaesthesia, and requires further investigation.

3.6.2 rmTBI impairs NSS performance

The Startle response, Limb Extension, Beam Walk, and Rotating Beam tasks are the four scored components of the NSS. A composite NSS score was generated for each group and trial. Prior to the first injury (baseline), animals were randomly assigned to the sham or rmTBI groups. No differences between groups was observed at baseline. In response to the first injury, average NSS scores significantly decreased compared to sham. Each subsequent injury also produced significant decreases in NSS performance compared to sham (**Figure 6**). Comparing rmTBI scores between trials revealed a significant decrease in score between the first injury and all subsequent injuries. In this regard, repeat injuries produced greater deficits than a single injury, but two, three, or four injuries were not cumulative. Sham animals NSS performance did not differ across the five trials, which suggests two important points. Firstly, the NSS test itself is easy enough that normal animals do not require learning trials and can consistently achieve near perfect scores after each trial. Additionally, this indicates that the restraint bag does not impair motor ability or confound the deficits observed in rmTBI animals.

Individual analysis of each task of the NSS test was performed. The Startle response is an innate reflex characterized by rapid activation of body muscles and an autonomic response to an acoustic stimulus (Prosser and Hunter, 1936; Szabo, 1964). rmTBI and sham animals did not display differences in this task of the NSS (**Figure 7A**). When suspended by the tail, rats exhibit a stereotypical forelimb extension where the animal extends the forelimbs toward the ground (Schaar et al., 2010). This response constitutes the Limb Extension task of the NSS, on which

rmTBI animals display significant impairment (**Figure 7B**). Both the Beam Walk and Rotating Beam tasks, tests of vestibulomotor function (Brooks and Dunnett, 2009), were also efficient at differentiating between sham and rmTBI animals (**Figure 7C, D**). The rotating beam was the least discriminatory of the four tasks, but arguably the most difficult task as sham animals did not generate perfect scores on average (**Figure 7D**).

3.6.3 rmTBI animals display anxiety-like behaviour

Anxiety disorders are reported in 10-70% of patients that have sustained a mTBI (Mooney and Speed, 2001; Rao and Lyketsos, 2002; Moore et al., 2006; Silver et al., 2009; Vaishnavi et al., 2009). The open-field test is commonly used in pre-clinical research to evaluate anxiety-like behaviour. Rodents have an innate preference for the periphery of an environment, called thigmotaxis, yet are naturally exploratory to novel environments and stimuli (Crawley, 1985, 1999). Anxiety-like behaviour is measured based on the duration of time spent in the periphery versus the centre of the field, where increased time in the perimeter or decreased time in the centre are indices of anxiety.

The current study represents the first investigation of open-field activity in a TBI model that does not use anaesthesia. On PID 1, the distance travelled in the centre and time spent exploring the centre of the open field was significantly reduced in rmTBI animals compared to sham controls (**Figure 8A, B**). The rmTBI animals displayed increased thigmotaxic behaviour, or increased time in the perimeter (**Figure 8B**). Sham and rmTBI animals did not differ in total distance travelled or speed of travel (**Figure 8C, D**), which is further evidence of affective behaviour. Injured animals were capable of the same exploratory activity as sham controls, but preferred the periphery due to anxiety-like symptoms following injury. Kwon and colleagues (2011) also identified anxiety-like behaviour only at 24 hours post-injury in a blast TBI model.

No deficits were observed at one or two months post-injury (Kwon et al., 2011). In contrast, no differences in open field behaviour were reported at 24 hours following lateral FPI (Shultz et al., 2011) or CCI (Almeida-Suhett et al., 2014).

On PID 7, a separate cohort of animals was tested in the open-field. Similar to PID 1, the rmTBI animals travelled the same total distance and with the same velocity as shams in the field (**Figure 9C, D**). However, after one week injured animals did not exhibit anxiety-like behaviour as measured by duration in the centre relative to the periphery (**Figure 9A, B**). No differences between injured and sham animals at PID 7 is in accordance with clinical cases, where the majority of human symptoms resolve spontaneously within 7-10 days post injury (McCrory et al., 2013). In experimental models, and in contrast to the findings presented herein, anxiety-like behaviour was observed at seven days after injury and persisted to 30 days after mild CCI with craniotomy (Almeida-Suhett et al., 2014). Persistent anxiety-like symptoms have also been identified 10 days after CCI (Yu et al., 2012a), and up to three months post-lateral FPI (Jones et al., 2008a; Johnstone et al., 2015). Conversely, other studies have shown no anxiety-like behaviour in the open-field test (Shultz et al., 2011; Washington et al., 2012; Cheng et al., 2014; Iliff et al., 2014; Mannix et al., 2014; Arain et al., 2015).

3.6.4 No risk-taking behaviour associated with ACHI rmTBI

Personality changes are a potential symptom of TBI (Greve et al., 2001; Tateno et al., 2003). Increased impulsivity or risk-taking behaviour has been observed following mTBI in a variety of clinical studies (Rapoport et al., 2003; Salmond et al., 2005; Tellier et al., 2009; Newcombe et al., 2011). The EPM is a widely used tool for evaluating experimental exploratory behaviour. Rodents exhibiting risk-taking behaviour will spend more time in the open arms of the apparatus.

The EPM was performed on PID 1 in the current study to determine the effects of rmTBI on risk-taking behaviour. rmTBI animals did not display risk-taking behaviour, as measured by frequency of entries into the open arms or duration of time in the open arms compared to shams (**Figure 10A, B**). These results are consistent with previous studies showing no differences between TBI and sham animals in the EPM (Zohar et al., 2011; Shultz et al., 2012a, 2015; Cheng et al., 2014; Arain et al., 2015; Nichols et al., 2016; Tan et al., 2016). In contrast, several investigations report risk-taking behaviour in TBI animals. Shultz *et al.* (2011) observed increased time in the open arm at 24 hours after mild lateral FPI, but not after 4 weeks recovery (Shultz et al., 2011). Risk-taking behaviour has additionally been reported following weight-drop injury (Pandey et al., 2009; Mannix et al., 2017), CCI (Washington et al., 2012; Johnson et al., 2013), and blast injury (Logsdon et al., 2014). Petraglia and colleagues offer interesting findings where single and repeat mTBIs caused decreased open arm entries 14 days after injury, which is consistent with other literature (Jones et al., 2008a; Kwon et al., 2011; Mychasiuk et al., 2015). However, rmTBI animals show increased time in the open arms, or risk-taking behaviour, one month and six months post-injury (Petraglia et al., 2014a). The finding of rmTBI causing impulsive or risk-taking-like behaviour has since been supported in an adolescent mouse model of TBI (Mannix et al., 2017).

3.6.5 rmTBI induces transient sensorimotor impairment

Balance and equilibrium deficits are another common feature of mTBI symptomology (Guskiewicz, 2011). In fact, balance problems occur after sports-related head injuries 30% of the time (Guskiewicz et al., 2000). The Rota-Rod test is commonly implemented in pre-clinical research to assess sensorimotor function as it requires motor coordination, sensory, and balance skills to remain on the rod as it gradually increases in speed of rotation over the test trial. Hamm

and colleagues (1994) were the first to utilize the Rota-Rod for TBI research, where they compared it to the previously established beam-balance and beam-walking tests. Following mTBI, only the Rota-Rod was sensitive enough to detect mTBI-induced motor dysfunction (Hamm et al., 1994). As such, the Rota-Rod was implemented in the current study, which is the first to assess sensorimotor function using the Rota-Rod in a TBI model that does not use anaesthesia.

Herein, sensorimotor function was evaluated one day before ACHI procedures (baseline), on PID 1, and again on PID 7 using the Rota-Rod. Animals were randomly assigned to treatment groups prior to baseline testing, and no differences between sham and rmTBI animals were observed (**Figure 11A-C**). On PID 1, rmTBI animals displayed significantly reduced performance on all measures including latency to fall, max speed achieved, and distance travelled (**Figure 11A-C**). This finding is in support of previous work that observed decreased Rota-Rod performance 24 hours after injury following lateral FPI (Maegele et al., 2015) or CCI (Yu et al., 2012a). Moreover, rmTBI animals displayed decreased performance compared to shams at PID 1-3 in adolescent mice (Mannix et al., 2017), as well as in adult mice (Mannix et al., 2014). Other rodent models of TBI have also reported impaired balance and motor coordination following weight-drop (Kane et al., 2012; Yan et al., 2013), and CCI (Longhi et al., 2005). Longhi and colleagues (2005) provided repeat injuries with three, five, or seven day inter-injury intervals. Single hit animals performed the same as sham, while all rmTBI groups displayed worse performance on the Rota-Rod (Longhi et al., 2005).

Animals were tested on the Rota-Rod a second time on PID 7 to evaluate motor recovery. The coordination and balance deficits identified 24 hour post-injury subsided by PID 7, where no differences in performance were observed between injured and sham animals (**Figure 11A-C**).

These findings are complimentary to those of Yu and colleagues (2012) who also performed a left parietal CCI injury and identified Rota-Rod deficits at one and three days post-injury, but reported recovery by PID 7 (Yu et al., 2012a). Other groups did not detect Rota-Rod impairment after various long-term time points after injury, including 1-4 weeks (Iliff et al., 2014), three months (Luo et al., 2014), or six months (Mouzon et al., 2014). Interestingly, rmTBI adolescent and adult animals that sustained seven impacts in nine days displayed persistently decreased performance for up to 3 months post-injury (Mannix et al., 2014, 2017).

3.6.6 No depressive-like behaviour in response to rmTBI

Depression is the most prominent neuropsychiatric symptom in patients with mTBI (Riggio and Wong, 2009; Silver et al., 2009), which typically presents within the first year after injury (Alderfer et al., 2005). Prevalence varies from 10-77% of cases dependent on the severity of injury (Rutherford, 1977; Jorge et al., 1993; Malkesman et al., 2013). The inability to perform normal daily tasks and other associated symptoms caused by head trauma likely exacerbate depressive symptoms (Pagulayan et al., 2008). Even worse, depression-like symptoms were found to be increased nearly three-fold in retired football players that had sustained rmTBI throughout their career (Guskiewicz et al., 2007). The Porsolt FST is commonly implemented in pre-clinical TBI research to evaluate depressive-like behaviour (Porsolt et al., 1978). Increased immobility compared to control animals is considered depressive-like behaviour (Porsolt et al., 1977, 1978).

In this study, the FST was conducted on a designated cohort of animals on PID 3-4. Activity thresholds (low, medium, high) in the swim apparatus were set based on previous literature (Jones et al., 2008a; Petraglia et al., 2014a; Shultz et al., 2015). No differences were observed in low threshold activity, or immobility, between sham and rmTBI animals. General

swim activity thresholds were similar between both groups (**Figure 12**). Previous research has shown findings consistent with those presented herein (Jones et al., 2008a; Shultz et al., 2011, 2015; Cheng et al., 2014; Mychasiuk et al., 2015; Nichols et al., 2016).

Depressive-like behaviour has been reported by previous studies. In single impact models, increased immobility was seen at seven days, and 1-3 months post-injury after mTBI using the weight drop model (Milman et al., 2005; Zohar et al., 2011). Washington and colleagues (2012) delivered injuries of increasing severity using a CCI model, which produced depressive-like symptoms across all severities at PID 21 (Washington et al., 2012). Other rmTBI research has found contrasting results to that of the present study. Petraglia and colleagues (2014) were the first to develop an awake injury model that does not require anaesthesia. Animals sustained 42 CCI impacts and displayed depressive-like behaviour one month post-injury (Petraglia et al., 2014a). Depressive-like behaviour was further reported following three mild lateral FPI at three months (Tan et al., 2016) and after five mild lateral FPI at eight weeks post-injury (Shultz et al., 2012a). Interestingly, these authors did not observe depression at 24 hours post, and only after several weeks (Shultz et al., 2012a). Considering the aforementioned studies that did find depressive-like behaviour, it is not surprising that the current study had no significant differences between sham and rmTBI animals. These previous studies all looked at more chronic time-points than PID 4, and delivered more repeat injuries with the exception of one study (Tan et al., 2016).

3.7 Chapter conclusions

The studies conducted in this Chapter demonstrate that rmTBI using the novel ACHI model produce acute neurological impairments and neurobehavioural sequalae similar to clinical cases. Injuries produced with the ACHI model alters consciousness acutely post-injury as

detected by the NSS. No apnea was observed, but loss of consciousness was identified in a subset of animals as measured by loss of toe pinch reflex and loss of righting reflex. Additional components of the NSS examine simple reflexive and motor tasks. Composite NSS scores made it clear that functional disturbances were present immediately post injury, and after each injury, compared to sham animals. Repeat injuries were shown to have a greater effect on NSS performance than only a single injury.

The NSS is designed to be simple, and quick in order to make an assessment of neurological function immediately within the first minute post injury. As such, the NSS is limited in addressing more complex neurobehavioural sequalae associated with clinical mTBI. To this end, a series of behavioural tests were performed. A behavioural battery including the open-field, EPM, and Rota-Rod was utilized to address anxiety, risk-taking behaviour, and motor coordination, respectively. The FST was also implemented to assay depressive-like behaviour. Results of these ethological investigations detected acute anxiety-like behaviour and transient motor coordination deficits in response to rmTBI. In both cases, the altered behaviours were detected at 24 hours after injury, but diminished after one week. The results presented in this Chapter provide evidence of functional and behavioural disturbances following injury in a novel rodent model of juvenile brain injury, which warrant further characterization of the ACHI procedure.

4. Biochemical analysis of Tau protein and related kinases following rmTBI

4.1 Background

Tau protein is the primary focus of this chapter. Upstream kinases were analyzed based on their association with tau. In this chapter, phosphorylation of tau protein, as well as GSK-3β and Akt, were assessed following ACHI rmTBI using Western Blotting.

Tau protein is a MAP that binds MTs to promote assembly and elongation, as well as stabilize existing MTs (Witman et al., 1976). Tau is a natively phosphorylated protein, yet hyper-phosphorylation causes reduced affinity and consequent MT disassembly, which has deleterious effects on cellular processes (Mandelkow and Mandelkow, 2012). Hyper-phosphorylated tau dissociates from MTs and aggregates into PHFs and subsequently into neurotoxic NFTs (Li et al., 2007; Cowan et al., 2010), which are the hallmark of CTE. This progressive neurodegenerative disease is thought to be the consequence of rmTBI (Corsellis et al., 1973; Omalu et al., 2005; McKee et al., 2009). However, it remains unknown whether tau protein aggregation presents the etiology of CTE, or is simply a marker of disease progression. As such, extensive interest in tau pathology following TBI has been undertaken and has resulted in the development of several experiment models (**Table 1**).

The current study investigates tau Ser202 phosphorylation. Ser202 is an epitope that is abnormally phosphorylated in PHF tau and commonly seen in AD brains (Goedert et al., 1993). The phosphorylation of Ser202, along with others, was found to be required for assembly of tau into filaments (Wang et al., 2007). Moreover, Ser202 is also found to be hyper-phosphorylated in CTE. In fact, the AT8 (Ser202/Thr205) and CP13 (Ser202) represent the antibodies regularly used by McKee and colleagues to diagnosis CTE post-mortem (McKee et al., 2009, 2013, 2014; Gavett et al., 2011; Stern et al., 2011; Goldstein et al., 2012; McKee and Robinson, 2014).

GSK-3β is a proline-directed serine/threonine kinase involved in glucose metabolism, cell division, differentiation, proliferation, and growth, as well as apoptosis. GSK-3β was investigated herein, as it is a primary tau kinase that is responsible for over one-third of all tau hyper-phosphorylation (Yang et al., 2013a). GSK-3β has been shown to phosphorylate tau protein at Ser202 (Wang et al., 2007). GSK-3β is a constitutively active kinase (Woodgett, 1990) that is regulated via inhibitory phosphorylation at Ser9, which was quantitated in the current study.

Akt is key regulator of cell survival, metabolism, protein synthesis, growth, and cell cycle regulation (Coffer and Woodgett, 1991; Jones et al., 1991; Kandel and Hay, 1999). The current study focuses on Akt as it is the most well-defined upstream regulator of GSK-3β (Cross et al., 1995). Akt requires phosphorylation of Ser473 for maximal kinase activity, and is therefore a key indicator of Akt activation (Scheid and Woodgett, 2003). Akt Ser473 phosphorylation was examined herein.

4.1.1 Chapter overview

Akt is a central regulatory kinase that is maximally activated when phosphorylated at Ser473. Akt is the principal regulator of GSK-3β via inhibitory phosphorylation at Ser9. GSK-3β is a primary tau kinase that phosphorylates tau at various sites, including Ser202. Tau is a MAP that binds to MTs to promote stability. Hyper-phosphorylation of tau causes decreased binding affinity that may result in release from MTs and subsequent microtubule destabilization. Hyper-phosphorylated tau aggregates into PHFs that further aggregate into neurotoxic NFTs, which are the pathological hallmark of neurodegenerative disorders such as AD and CTE. In response to brain injury, a cascade of altered neurometabolic activity is set off, which may result in tau hyper-phosphorylation. This chapter uses Western blotting to investigate relative changes

in the phosphorylation of tau, GSK-3β, and Akt at Ser202, Ser9, and Ser473, respectively, following rmTBI in juvenile rats. The hippocampal sub-regions, DG and CA, and the cortex (CTX) were collected from the ipsilateral and contralateral, to injury site, hemispheres on PID 7.

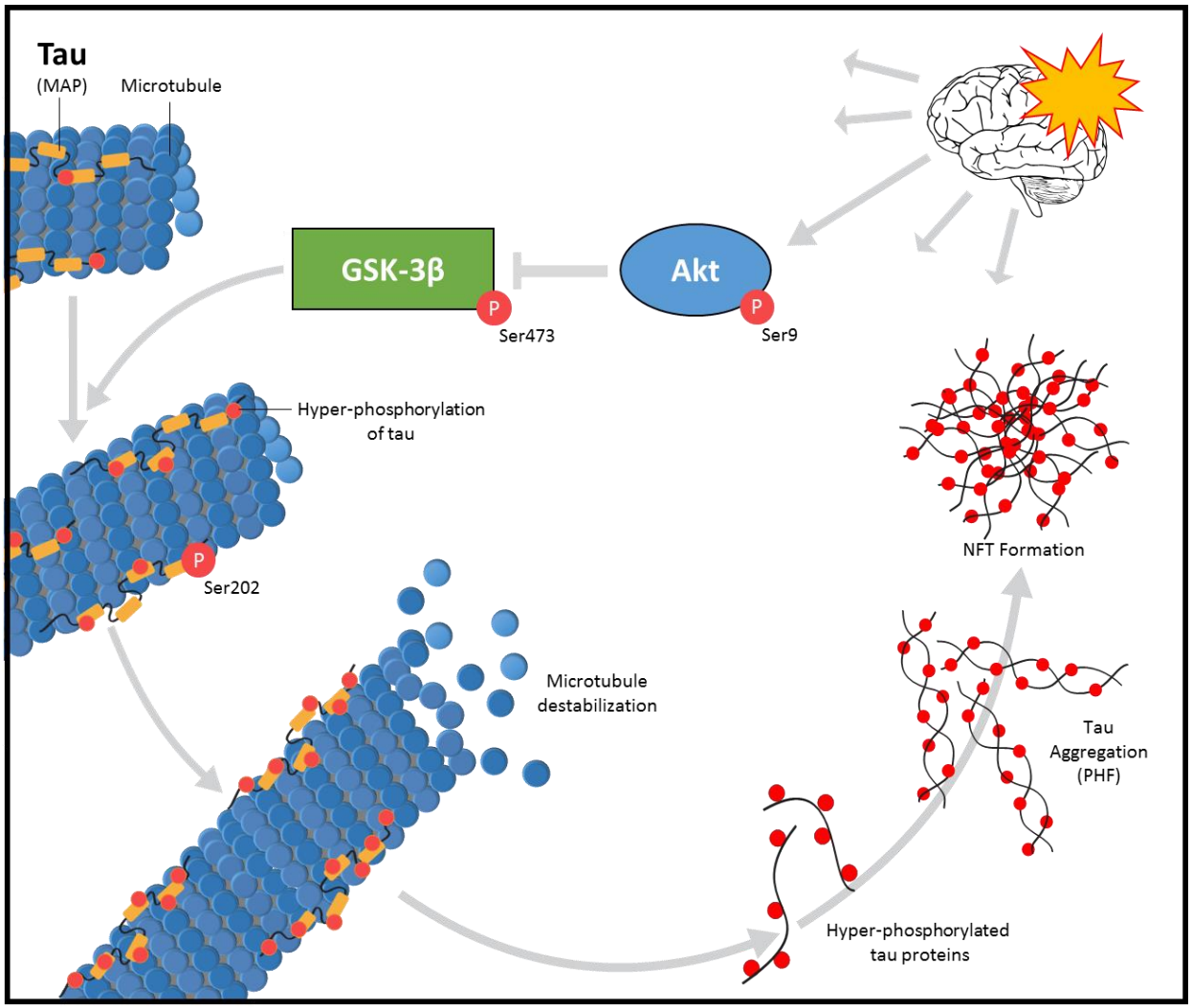

Figure 13. Chapter overview. Tau, a microtubule-associated protein (MAP), binds to microtubules to promote stability. Hyper-phosphorylation of tau may result in release from microtubules and subsequent microtubule destabilization. Hyper-phosphorylated tau aggregates into paired helical filaments (PHF) that further aggregate into neurotoxic neurofibrillary tangles (NFT). Akt is a central regulatory kinase that is maximally activated when phosphorylated at Ser473. Akt is the principal regulator of glycogen synthase kinase-3β (GSK-3β) via inhibitory phosphorylation at Ser9. GSK-3β is a primary tau kinase that phosphorylates tau at Ser202. In response to brain injury, a cascade of altered neurometabolic activity is set off, which may result in tau hyper-phosphorylation. This chapter investigates changes to tau, GSK-3β, and Akt at Ser202, Ser9, and Ser473, respectively, following rmTBI in juvenile rats.

4.2 Biochemical analysis

4.2.1 Tissue preparation

Rats were deeply anaesthetized with inhaled isoflurane (Abbott Laboratories, North Chicago, IL), immediately decapitated and the head placed on ice. The brain was removed and kept in cold 0.1M Phosphate-buffered saline (PBS). The brain was then bisected on ice. The DG was crudely separated from the CA following the procedure previously described (Hagihara *et al.*, 2009). Both regions were quickly frozen in liquid N_2. A portion of overlying CTX was also dissected and frozen in liquid N_2. All samples were stored at -80°C until homogenization.

Lysis buffer [20mM Tris pH8, 137mM NaCl, 0.1% (v/v) NP-40, 10% (v/v) glycerol, 2mM ethylenediaminetetraacetic acid (EDTA), 1X Halt™ phosphatase and protease inhibitor (100X) (ThermoScience, Rockford, IL, USA)] was added to each sample at 10mL/g of tissue. The samples were then immediately sonicated (Fisher Scientific, Pittsburgh, PA) 4 times for 5 seconds with a 15 second inter-sonication interval. The samples were centrifuged at 14,000 *g* for 15 minutes at 4°C in a microcentrifuge (Fisher Scientific). Supernatants were obtained and stored at -80°C until later processing.

4.2.2 Protein quantification

A bicinchoninic acid (BCA) protein assay was used to quantify total protein concentration using a commercially available kit (BCA Protein Assay Kit, Pierce, Rockford, Illinois, USA). A bovine serum albumin (BSA) standard curve was performed from a 2 mg/mL BSA standard stock ranging from 0.03125 mg/mL – 2 mg/mL. All brain lysates were diluted to 1:50 for protein concentration detection. Working reagent was prepared by mixing BCA Reagent A with BCA Reagent B, and 200 µL of working reagent was added to all standard and sample wells in a 96-well microtitre plate. The plate was then incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm in the VersaMAX plate reader (Molecular Devices,

Sunnyvale, CA, USA) and analyzed with Softmax Pro 5.2 (Molecular Devices). The curve fit for the generated standard curve was a log-log fit and the concentration of protein (μg/mL) was determined from this curve.

4.2.3 Western blotting

Samples were diluted in 5X Reducing Sample Buffer [0.25M Dithiothreitol (DTT), 5% (v/v) sodium dodecyl sulfate (SDS), 0.2M Tris pH 6.8, 1.5% (w/v) Bromophenol Blue, 37.5% (v/v) Glycerol], heated at 95°C for 5 minutes, cooled to room temperature and then used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 15μg of protein was loaded along with 7 μL of kaleidoscope molecular weight markers (BioRad), separated on a 17% separating gel [Milli Q H_2O, 0.39M Tris pH 8, 10% (w/v) Acrylamide, 0.2% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS), 0.4 μL/mL tetramethylethylenediamine (TEMED) (BioRad)] at 130V in an electrophoresis tank (BioRad) containing 1X SDS-PAGE Running Buffer [0.025M Tris, 0.192M Glycine, 0.1% (v/v) SDS]. The proteins were transferred in 1X Transfer Buffer [0.025M Tris, 0.192M Glycine, 0.1% (v/v) SDS, 20% (v/v) Methanol] at 4°C onto Polyvinylidene Fluoride (PVDF) membranes (Perkin Elmer, Boston, MA, USA) at 40V overnight. The membranes were stained with Ponceau S (Sigma, Saint Louis, Missouri) in order to determine transfer efficiency.

Different blocking conditions, primary antibody concentrations and secondary antibody concentrations were utilized dependent on the protein of interest (**Table 4**). Membranes were either blocked in 5% Skim Milk [5% (w/v) Skim Milk (Difco™) or 5% BSA [5% (w/v) BSA (Sigma), 0.05% (v/v) Tween-20 in 1X Tris Buffered-Saline (TBS)] for 1 hour at room temperature. Membranes were probed with α-phospho-Tau (Ser202) (Cell Signaling Technology, Danvers, MA), α-Tau-5 (total Tau) (Thermo Scientific, Rockford, IL), α-phospho-GSK-3β

(Ser9) (Cell Signaling), α-GSK-3β (Cell Signaling), α-phospho-Akt (Ser473) (Cell Signaling), α-Akt (pan) (Cell Signaling), and α-GAPDH (loading control; Cell Signaling) (**Table 4**)

Membranes were incubated in either goat α-rabbit IgG (H+L) horseradish peroxidase (HRP)-conjugate [Chemicon (Millipore, Temecula, CA)] or goat α-mouse IgG (H+L) HRP-conjugate (KPL, Gaithersburg, MD) for 1 hour at room temperature (**Tables 4**). Membranes were washed with a TBS- or PBS-based 0.05% Wash Buffer [0.05% (v/v) Tween-20 in 1X TBS or PBS] three times for 5 minutes between blocking and primary antibody incubation, between primary and secondary antibody incubations, and after the secondary antibody incubations (**Tables 4).**

Table 4. Summary of Western Blotting conditions.

Antibody	Blocking buffer	Primary antibody			Secondary antibody
		Dilation	Buffer	Incubation	
monoclonal rabbit α-phospho-Tau (Ser202)	5% (w/v) BSA	1:1000	5% (w/v) BSA	Overnight at 4°C	1:10 000
monoclonal mouse α-Tau-5	5% (w/v) Skim milk	1:1000			1:10 000
monoclonal rabbit α-pGSK-3β (Ser9)	5% (w/v) BSA	1:1000			1:10 000
monoclonal rabbit α-GSK-3β	5% (w/v) Skim milk	1:1000			1:10 000
monoclonal rabbit α-pAkt	5% (w/v) BSA	1:8000			1:10 000
monoclonal rabbit α-Akt	5% (w/v) Skim milk	1:10 000			1:10 000
monoclonal rabbit α-GAPDH	5% (w/v) Skim milk	1:15 000	5% (w/v) Skim milk		1:10 000

Membranes were subject to stripping in Stripping Buffer [62.5mM Tris HCl pH 6.7, 2% (v/v) SDS and 1% (v/v) of β-mercaptoethanol (BioRad)] at 50°C for 30 minutes, washed,

blocked and reprobed for the next protein of interest. Each membrane was striped no more than twice. Western blots were visualized by Clarity™ enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA) and imaged with a G:Box Chemi-XR5 using GENESys software (Syngene, Cambridge, UK). Blot images were quantified by densitometric analysis using local background subtraction in the QuantityOne Software (Bio-Rad). The relative levels of total protein were normalized to GAPDH, while the relative phospho-protein levels were normalized to the corresponding total protein.

4.2.4 Statistical analysis

Two-tailed Student's *t*-test were used to determine statistical differences in expression levels using Statistica 7.0 analytical software (Statsoft Inc., Tulsa, OK, USA) and Excel 2007 (Microsoft, Richmond, VA, USA). Data are presented as Mean ± SEM for both Sham and rmTBI. Differences were considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 rmTBI does not alter tau phosphorylation

On PID 7, tau protein phosphorylation at Ser202 was quantitated as the ratio of phospho-tau (pS202) to total tau (Tau-5). No significant differences between sham and rmTBI animals were identified in the ipsilateral ($t_{(12)} = 1.25$, $p = 0.24$; **Figure. 14A**) or contralateral hemispheres ($t_{(12)} = 0.52$, $p = 0.61$; **Figure. 14A**) of the DG. Similar findings were found in the ipsilateral ($t_{(11)} = -0.12$, $p = 0.91$; **Figure. 14C**) and contralateral ($t_{(12)} = -0.51$, $p = 0.62$; **Figure. 14C**) CA, as well as in the ipsilateral ($t_{(12)} = -1.01$, $p = 0.33$; **Figure. 14E**) and contralateral ($t_{(12)} = -0.65$, $p = 0.53$; **Figure. 14E**) CTX. Total tau protein (Tau-5) levels were unchanged with the exception of the ipsilateral CA where rmTBI significantly decreased Tau-5 compared to sham ($t_{(11)} = 2.56$, $p = 0.03$; **Appendix A**).

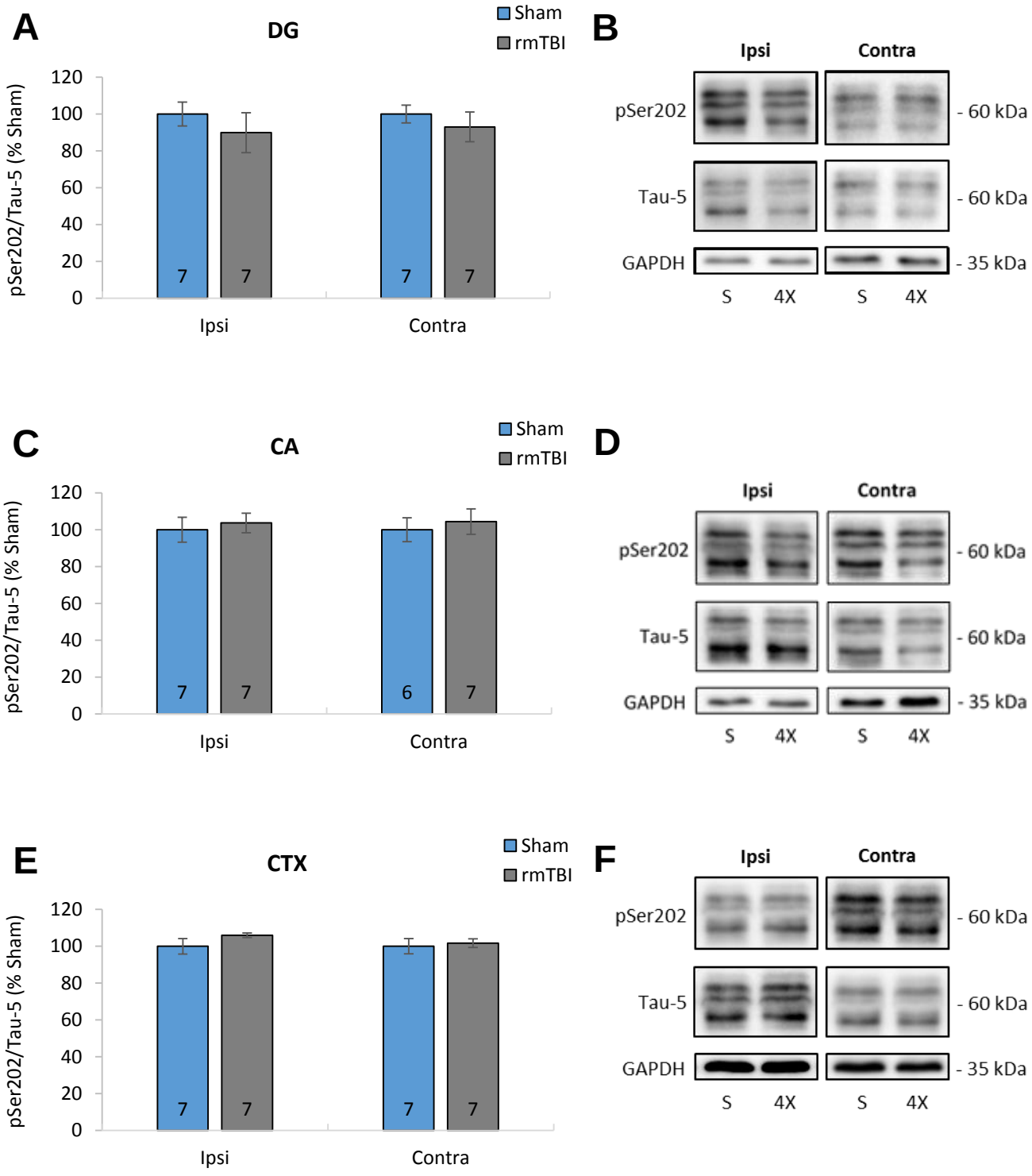

Figure 14. rmTBI does not alter tau protein phosphorylation at Ser202. Western blot analysis of pS202 on tau protein in juvenile sham and rmTBI rats. Relative amounts of phosphorylation was calculated as a ratio of pS202 to total tau (Tau-5), and presented as a percentage of sham. Tau-5 was normalized to GAPDH. No changes in pS202 were observed in the ipsilateral (ipsi) or contralateral (contra) DG (A), CA (C), or CTX (E). Representative Western Blots for pS202, Tau-5 and GAPDH in the DG (B), CA (D), and CTX (F). Data is expressed as Mean ± SEM. Number of samples are denoted in the respective bars. Sham: S; rmTBI: 4X.

4.3.2 rmTBI does not alter GSK-3β phosphorylation

On PID 7, GSK-3β protein phosphorylation at Ser9 was quantitated as the ratio of phospho-GSK-3β (pGSK-3β) to total GSK-3β. No significant differences between sham and rmTBI animals were identified in the ipsilateral ($t_{(12)}$ = -0.33, p = 0.75; **Figure. 15A**) or contralateral hemispheres ($t_{(12)}$ = -0.06, p = 0.95; **Figure. 15A**) of the DG. Similar findings were found in the ipsilateral ($t_{(12)}$ = -1.78, p = 0.10; **Figure. 15C**) and contralateral ($t_{(12)}$ = -1.47, p = 0.17; **Figure. 15C**) CA, as well as in the ipsilateral ($t_{(12)}$ = -1.02, p = 0.33; **Figure. 15E**) and contralateral ($t_{(12)}$ = -1.27, p = 0.23; **Figure. 15E**) CTX. No changes in total GSK-3β levels were detected (data not shown).

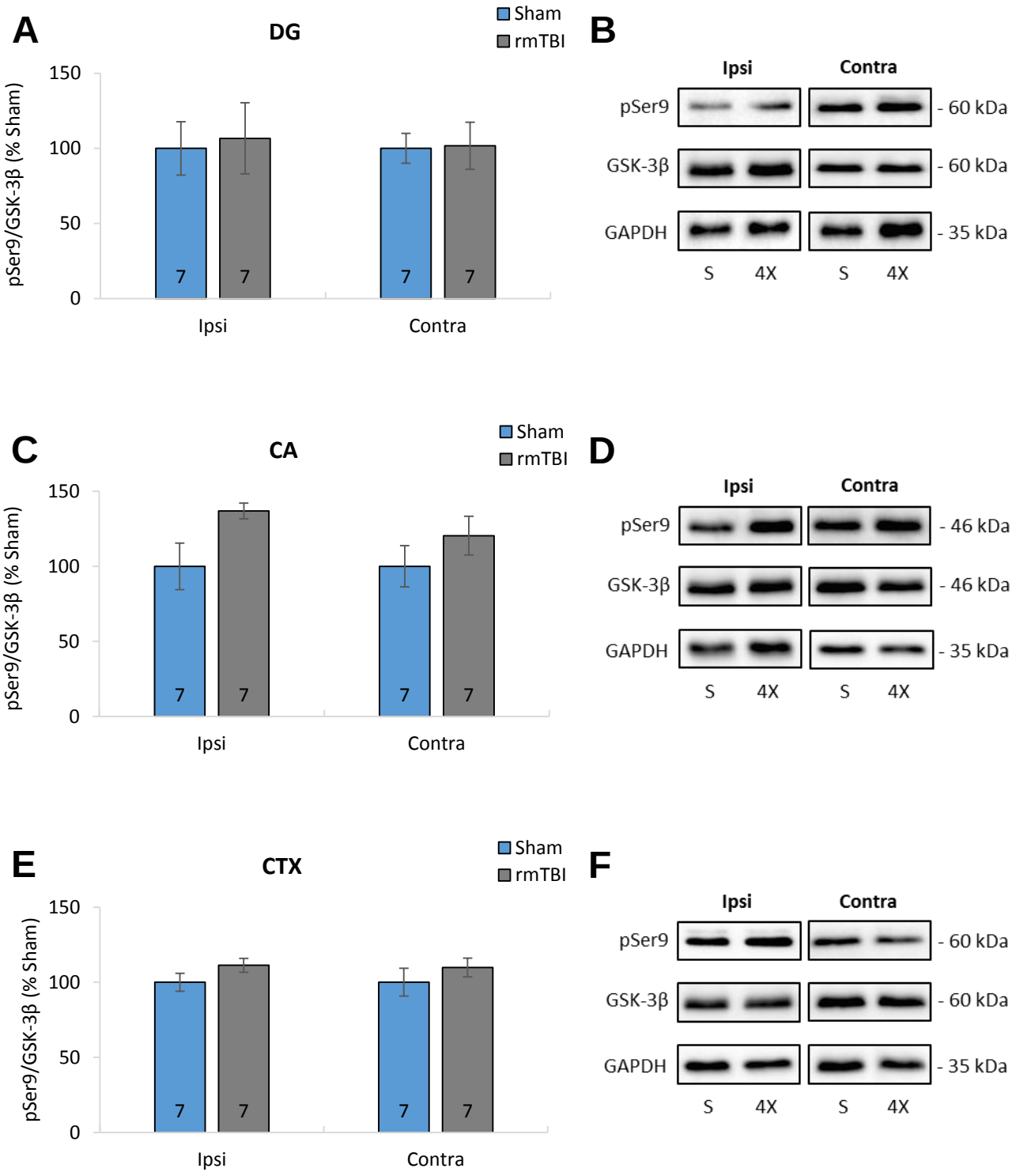

Figure 15. rmTBI does not alter GSK-3β protein phosphorylation at Ser9. Western blot analysis of pS9 on GSK-3β protein in juvenile sham and rmTBI rats. Relative amounts of phosphorylation was calculated as a ratio of pS9 to total GSK-3β, and presented as a percentage of sham. Total GSK-3β was normalized to GAPDH. No changes in pS9 were observed in the ipsilateral (ipsi) or contralateral (contra) DG (A), CA (C), or CTX (E). Representative Western Blots for pS9, GSK-3β and GAPDH in the DG (B), CA (D), and CTX (F). Data is expressed as Mean ± SEM. Number of samples are denoted in the respective bars. Sham: S; rmTBI: 4X.

4.3.3 rmTBI does not alter Akt phosphorylation

On PID 7, Akt protein phosphorylation at Ser473 was quantitated as the ratio of phospho-Akt (pAkt) to total Akt. No significant differences between sham and rmTBI animals were identified in the ipsilateral ($t_{(12)}$ = -0.94, p = 0.37; **Figure. 16A**) or contralateral hemispheres $t_{(12)}$ = -0.15, p = 0.88; **Figure. 16A**) of the DG. Similar findings were found in the ipsilateral ($t_{(12)}$ = -1.25, p = 0.24; **Figure. 16C**) and contralateral ($t_{(11)}$ = -1.60, p = 0.14; **Figure. 16C**) CA, as well as in the ipsilateral ($t_{(12)}$ = -0.48, p = 0.64; **Figure. 16E**) and contralateral ($t_{(12)}$ = -0.06, p = 0.95; **Figure. 16E**) CTX. No changes in total Akt levels were detected (data not shown).

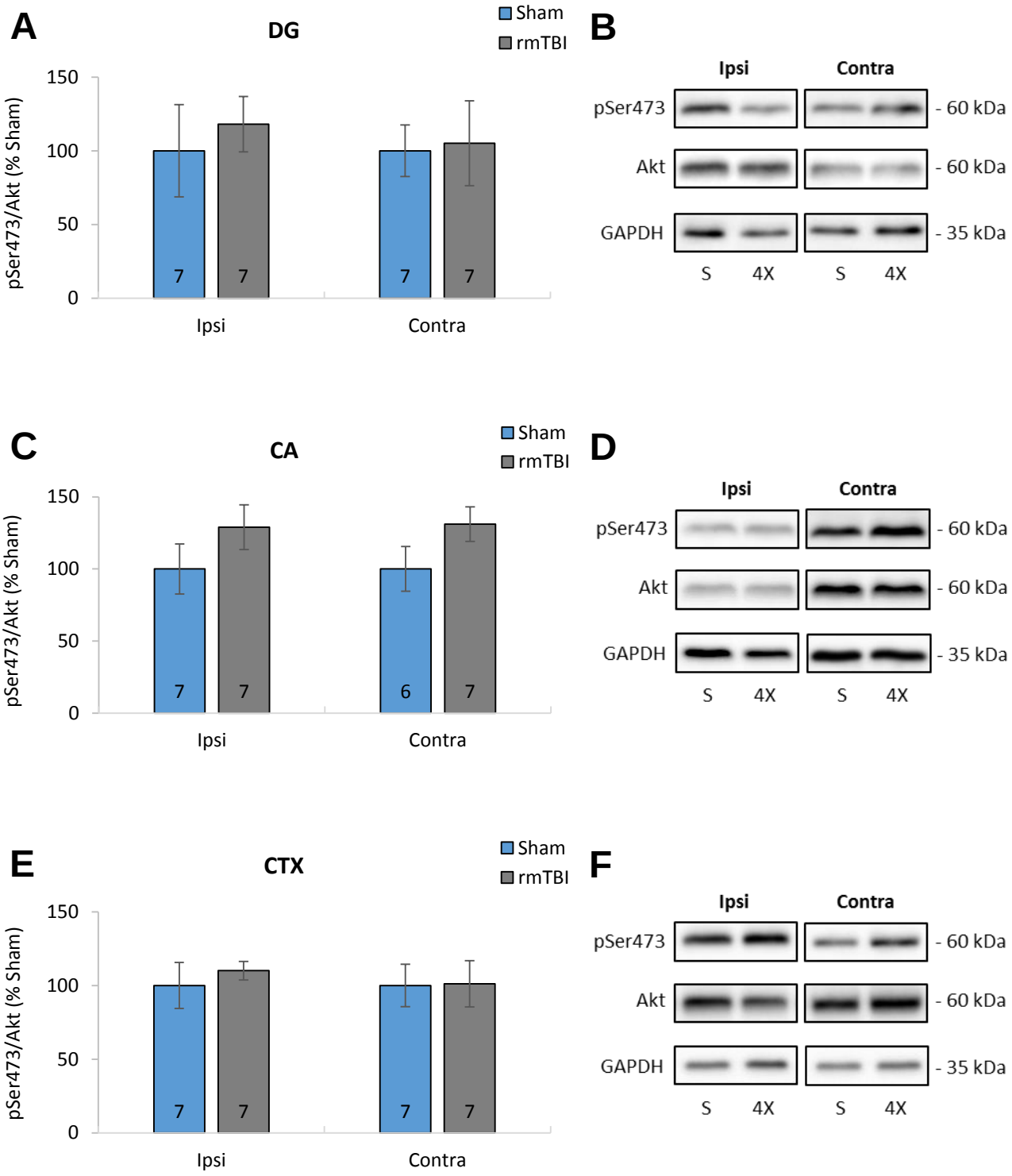

Figure 16. rmTBI does not alter Akt protein phosphorylation at Ser473. Western blot analysis of pS473 on Akt protein in juvenile sham and rmTBI rats. Relative amounts of phosphorylation was calculated as a ratio of pS473 to total Akt, and presented as a percentage of sham. Total Akt was normalized to GAPDH. No changes in pS473 were observed in the ipsilateral (ipsi) or contralateral (contra) DG (A), CA (C), or CTX (E). Representative Western Blots for pS473, Akt and GAPDH in the DG (B), CA (D), and CTX (F). Data is expressed as Mean ± SEM. Number of samples are denoted in the respective bars. Sham: S; rmTBI: 4X.

4.4 Discussion

4.4.1 Tau phosphorylation at Ser202 is not altered by rmTBI

In the present study, tau protein phosphorylation at Ser202 was evaluated following rmTBI. Tau protein is of significant interest in TBI research, as it is the defining characteristic of CTE, which is proposed to be the consequence of rmTBI. Ser202, specifically, was investigated as this epitope is actively used in the diagnosis of CTE. rmTBI animals did not display changes to relative levels of pS202 (pTau) in any of the brain regions investigated as determined by the ratio of pTau to total tau. Both the ipsilateral and contralateral DG, CA, and CTX did not display altered tau phosphorylation in rmTBI animals as compared to shams on PID 7 (**Figure 14A-F**).

As is evident in **Table 1**, and in contrast to the findings presented herein, tau pathology has previously been identified in experimental models. Tau pathology has been reported on several occasions after a single TBI (of varying severities) in a variety of experimental models that utilize a craniotomy to deliver injury. These studies will not be discussed in detail due to the confounding nature of craniotomy, but have reported elevated cleaved-tau (Gabbita et al., 2005), increased total tau (Rostami et al., 2012; Tran et al., 2012), accumulation of tau breakdown products (Liu et al., 2011), and increased pTau levels (Hoshino et al., 1998; Tran et al., 2011; Yu et al., 2012b; Hawkins et al., 2013). In models of CHI, a single impact is still sufficient to induce changes to tau protein. Smith and colleagues (1999), using miniature pigs and a non-impact rotational acceleration model of injury, report tau accumulation throughout the brain at 3-10 days post trauma, but not at 24 hours (Smith et al., 1999). In weight-drop models, increased pTau was identified as early as one hour in serum (Liliang et al., 2010) and at four hours in the brain (Genis et al., 2000), but had decreased back sham levels by 24 hours in both cases. Although single impact models, both of the previously mentioned studies also tested for tau

phosphorylation at PID 7, and saw no differences compared to sham (Genis et al., 2000; Liliang et al., 2010), which is in agreement with the present study. Additional investigations of tau protein after a single injury detected elevated pTau at two weeks (Goldstein et al., 2012), 28 days (Iliff et al., 2014), and one month post-injury (Huber et al., 2013; Perez-Polo et al., 2015). Elevated tau was also identified after 2.5 months after blast injury (Kovesdi et al., 2012).

Following head trauma, the brain is putatively in a state of vulnerability in which a second injury may exacerbate the damage. This notion is supported by clinical evidence where patients that have sustained multiple injuries displayed increased and prolonged deficits. Pre-clinical findings further strengthen the argument as repeated injuries have been shown to produce deficits not observed after a single injury. In regard to tau protein, rmTBI is thought to underlie CTE. Several experimental models of rmTBI have since examined tau protein considering this link to CTE. Kane and colleagues (2012) delivered one injury per day for five consecutive days using a modified weight drop model. Western blotting revealed increased pTau in the cortex one month after the last injury (Kane et al., 2012). Another laboratory also delivered five injuries (in nine days; CCI) and identified increased pTau 21 days post. Interestingly, tau pathology was only observed in the rmTBI animals, and not after a single hit (Ojo et al., 2013). Increased pTau has further been observed after two, three, or six repeat injuries at various time-points post-injury ranging from six hours to six months (Luo et al., 2014; Namjoshi et al., 2014; Zhang et al., 2015; Lucke-wold et al., 2016). Petraglia and colleagues (2014), using their awake CCI mouse model, delivered 42 impacts in six days. Immunohistochemistry revealed elevated levels of pTau at seven days post-injury, which was still evident at six months (Petraglia et al., 2014b).

In contrast, a collection of studies have failed to detect changes in tau protein after injury. Ojo and colleagues (2014) delivered a mTBI using a CCI model, and detected no changes to

Ser202 phosphorylation at 34 days post-injury (Ojo et al., 2014). In accordance with the current study, a number of rmTBI models also failed to detect changes to tau phosphorylation at 24 hours (Bolton and Saatman, 2014; Gama Sosa et al., 2014; Wang et al., 2017), seven days (Wang et al., 2017), 10 weeks (Xu et al., 2016), or six months (Mannix et al., 2013; Mouzon et al., 2014) after head trauma. The majority of literature, however, provides compelling evidence that TBI causes increased phosphorylation of tau protein in rodents. Increased pTau was reported across a wide range of brain regions and time-points. Variability is likely a function of injury severity, time-point post-injury, and number of impacts delivered. Many of the rmTBI models described investigate time-points greater than PID 7 (Kane et al., 2012; Ojo et al., 2013; Luo et al., 2014; Petraglia et al., 2014b; Zhang et al., 2015; Lucke-wold et al., 2016), which could explain the lack of differences presented herein. In addition to assessing a chronic time-point, the inclusion of more injuries may be necessary to induce tau pathology in an awake model (Petraglia et al., 2014b).

4.4.2 Phosphorylation of GSK-3β Ser9 is not affected by rmTBI

GSK-3β is a primary tau kinase that phosphorylates tau at a number of serine/threonine sites, including Ser202. GSK-3β is associated with hyper-phosphorylated tau deposits in the AD brain (Yamaguchi et al., 1996) and experimental overexpression promotes tau protein phosphorylation (Brownlees et al., 1997). GSK-3β activity is turned off by inhibitory phosphorylation at Ser9, which has been linked to neuroprotective outcomes (Cross et al., 2001; Li et al., 2002; Hongisto et al., 2003). In the current study, phosphorylation of GSK-3β at Ser9 was evaluated on PID 7 after rmTBI using Western blotting. rmTBI animals did not display changes to relative levels of pS9 (pGSK-3β) in any of the brain regions investigated as determined by the ratio of pGSK-3β to total GSK-3β. Both the ipsilateral and contralateral DG,

CA, and CTX did not display altered GSK-3β phosphorylation in rmTBI animals as compared to shams on PID 7 (**Figure 15**). Additionally, no changes in total GSK-3β levels were detected.

The results presented herein are in agreement with previous studies. Tran and colleagues (2012) delivered CCI TBI to transgenic-AD model mice, which did not alter GSK-3β phosphorylation 24 hours post-injury as assessed by Western blotting in hippocampal lysates. Moreover, there was no Ser9 immunoreactivity below the impact site, nor the ipsilateral and contralateral CA1 (Tran et al., 2012). Dash and colleagues (2011) report a transient increase in pGSK-3β at three days after CCI. However, no changes were observed at 30 minutes, three hours, one day, or 14 days compared to sham (Dash et al., 2011). Similarly, increased pGSK-3β was reported at 24 hours and three days post CCI, but was not significant at seven days post-injury (Zhao et al., 2012). In contrast to the present study, increased pGSK-3β was detected at PID 8 and PID 30 following CCI (Zhang et al., 2015), and as early as 3, 12, and 24 hours after weight drop impact (Shapira et al., 2007). Again, Dash and colleagues (2011) identified elevated pGSK-3β in hippocampal tissue three days post-CCI, but not at early time-points or after 14 days (Dash et al., 2011). Collectively, the literature shows little consistency in regard to GSK-3β phosphorylation at Ser9 after head injury. However, previous findings, in addition to those presented herein, suggest that brain injury may alter Ser9 phosphorylation, but in a limited window after trauma. Further investigation is required.

GSK-3β dysregulation is linked to the pathogenesis of several disorders, including Alzheimer's (Lei et al., 2011; Avrahami et al., 2013), cancer (Sokolosky et al., 2014; Azoulay-Alfaguter et al., 2015), diabetes (Gao et al., 2011), and neuroinflammation (Li et al., 2013), such that GSK-3β is often targeted for therapeutic intervention. Indeed, the majority of TBI-related research has examined the inhibition of GSK-3β as a putative therapy after brain injury. This is

based on the well-established finding that inhibition of GSK-3β has been linked to neuroprotective outcomes (Cross et al., 2001; Li et al., 2002; Hongisto et al., 2003) and that its inhibition suppresses glutamate excitotoxicity in neuronal culture (Liang and Chuang, 2007). Lithium, for example, is a direct inhibitor of GSK-3β (Klein and Melton, 1996) and indirectly inhibits GSK-3β by activation of Akt (Beaulieu et al., 2004), PKA (Jope, 1999), and PKC (Kirshenboim et al., 2004). In rodent models of TBI, inhibition of GSK-3β with lithium reduced TBI-induced brain lesions, decreased neurodegeneration, suppressed TBI-induced neuroinflammation, attenuated BBB breakdown, improved motor coordination after TBI, and alleviated TBI-induced anxiety-like behaviours (Yu et al., 2012a). Additionally, GSK-3β inhibition by lithium improved hippocampal-dependent learning and memory, reduced CA3 neuronal loss (Dash et al., 2011), and reduced TBI-induced depressive behaviour (Shapira et al., 2007).

4.4.3 rmTBI does not alter phosphorylation of Akt at Ser473

Akt is a central regulator in a variety of cellular signaling mechanisms, notably cell survival and apoptosis. Akt is the most well-defined upstream kinase of GSK-3β (Cross et al., 1995). Phosphorylation by Akt at Ser9 inactivates GSK-3β, and is proposed to underlie much of the cell survival effects attributed to Akt (Hers et al., 2011). Phosphorylation at Ser473 of Akt is required for maximal activity, and is therefore a key indicator of Akt activation (Scheid and Woodgett, 2003). The present study utilized Western blotting to evaluate Akt Ser473 phosphorylation on PID 7 after rmTBI. ACHI injured animals did not display changes to relative levels of pS473 (pAkt) in any of the brain regions investigated as determined by the ratio of pAkt to total Akt. Both the ipsilateral and contralateral DG, CA, and CTX did not display altered Akt

phosphorylation in rmTBI animals as compared to shams on PID 7 (**Figure 16**). Additionally, no changes in total Akt levels were detected.

The findings presented herein are in accordance with previously reported findings. Gao and colleagues (2016) did not detect altered Akt phosphorylation or changes to total levels of Akt at 24 hours post-injury in the ipsilateral cortex (Gao et al., 2016). On PID 7, relative pAkt levels were not significantly different than sham animals after CCI, but were elevated at four hours after injury (Zhao et al., 2012). Indeed, transient increases in Ser473 phosphorylation at four hours have been reported on several occasions before decreasing to control levels at 24 hours post-injury (Noshita et al., 2002; Park et al., 2012; Zhao et al., 2014). In contrast, Rubovitch and colleagues (2010) identified increased Ser473 phosphorylation in the hippocampus following weight-drop injury that was significant at 1, 24, and 72 hours post (Rubovitch et al., 2010), which was further supported by increased hippocampal pAkt at 24 hours (Zhu et al., 2014; Wang et al., 2017) and 48 hours (Du et al., 2016) post-injury. Further disparity in the literature was identified in juvenile rats that presented decreased Akt phosphorylation at 24 h after CCI (Jenkins et al., 2002). Wang and colleagues (2013) also report decreased pAkt in response to CCI at PID 7 compared to shams (Wang et al., 2013). The findings discussed herein, suggest a trend that Akt phosphorylation at Ser473 peaks early, and transiently post-injury, but does not persist. The findings of the current study corroborate previous literature.

4.5 Chapter conclusions

In this Chapter, tau protein phosphorylation at Ser202 was investigated using Western Blotting. No changes to pSer202 were idenfitied on PID 7 in the DG, CA, or CTX of either the ipsilateral or contralateral cortex. GSK-3β, a primary tau kinase, was also investigated. GSK-3β

is regulated by inhibitory phosphorylation at Ser9. No changes in relative Ser9 phosphorylation was identified in any of the regions or hemispheres examined following rmTBI. Akt is found upstream of GSK-3β and is the key regulator of GSK-3β activity. Akt is maximally activated when phosphorylated at Ser473. Herein, pSer473 did not differ between sham and rmTBI animals one week post-injuyr. Previous studies identified changes in tau, GSK-3β, and Akt using a variety of TBI models and time-points post-injury. The null results presented in the current study may, in part, be a factor of the post-injury time-point used, or of the ACHI model injury severity. In general, altered tau phosphorylation was readily appartent at more chronic time-points post-injury, while GSK-3β and Akt phosphorylation appear to peak acutely and transiently.

5. General Discussion

The present study has developed a novel model of CHI that was used to deliver rmTBI to unanaesthetized juvenile rats. The ACHI model boasts two primary benefits in comparison to other previously developed experimental models. Impacts are delivered to fully conscious, restrained animals without the need of anaesthesia, which is known to be neuroprotective. In addition, no craniotomy surgery is performed. The absence of anaesthesia and craniotomy avoid unnecessary confounds and likely make the ACHI model more representative of clinical mTBI. Furthermore, the lack of recovery from anaesthesia and surgery allows the researcher to make an immediate assessment of neurological function following each injury.

5.1 Ethology

NSS testing demonstrated that ACHI causes acute impairment to consciousness following each injury. No apnea was observed, but the toe pinch reflex and righting reflex was lost in a subset of animals. CC were observed in over half of the injured animals, and represents a novel finding in awake TBI models that requires further investigation. The NSS also identified significant deficits in gross motor ability following injury. rmTBI animals displayed reduced performance after each of the four injuries compared to sham controls. Interestingly, NSS performance was significantly lower after repeat injury than after the first, single injury. This further contributes to the notion of the brain being in an enhanced state of vulnerability after injury and that additional injury worsens outcomes, at least within a 24 hour window as was presented herein.

A mTBI is a functional disturbance, which may, or may not involve loss of consciousness (McCrory et al., 2013). The findings presented herein show that the ACHI model acutely alters consciousness and produces functional impairments as measured by the NSS immediately post-

injury. This is a critical first step in the characterization of this novel model and supports the utility of this model for future investigations. The NSS was designed to be simple and rapidly executed in order to make an immediate assessment of neurologic function after injury. However it does not address more complex cognitive, motor, and emotional changes associated with clinical mTBI (Gibb and Kolb, 1998; Carroll et al., 2004; McCrea et al., 2009; McCrory et al., 2013).

To complement the NSS, and to further investigate if the ACHI model induces neurobehavioural sequalae similar to that of the clinical population, a series of behavioural tests were performed. Open-field testing found that rmTBI induces anxiety-like behaviour. Injured animals spent significantly increased time in the periphery and less time in the centre of the field, an index of anxiety, compared to sham animals. This affective behaviour was present one day post-injury, but was not found identified one week post. The EPM was used to detect risk-taking, or impulsivity, a common symptom of mTBI (Rapoport et al., 2003; Salmond et al., 2005; Tellier et al., 2009; Newcombe et al., 2011). rmTBI animals did not display risk-taking behaviour, as measured by frequency of entries and time spent in the open arms compared to shams. The Rota-Rod test was used to assess motor coordination and balance, which is a common deficit observed following sports-related head injuries (Guskiewicz et al., 2000). rmTBI animals exhibited significant impairment in performance 24 hours after injury, which recovered after one week. Depression is the most prominent neuropsychiatric symptom in patients with mTBI (Riggio and Wong, 2009; Silver et al., 2009). To evaluate depression-like symptoms in rmTBI animals, the FST was performed, which did not detect altered behaviour in rmTBI animals. Collectively, the ethological tests presented in the current study provide

evidence of functional and behavioural disturbances following rmTBI in a novel rodent model of juvenile brain injury.

5.2 Biochemical analysis

CTE is a progressive neurodegenerative disorder characterized by NFTs consisting of insoluble tau protein. rmTBI is proposed to be the underlying cause of CTE, yet the exact mechanisms and threshold for pathology is currently unknown. Tau protein pathology is well-defined in a variety of other neurodegenerative disorders, although whether tau aggregates represent the causative agent or are merely markers of disease remains to be elucidated. Tau protein phosphorylation at Ser202 was investigated in the present study. Ser202 was selected since it has previously been identified in CTE brains (McKee et al., 2009). No changes were identified at PID 7 in the DG or CA hippocampal sub-regions, or in the overlying CTX in neither the ipsilateral or contralateral hemispheres. Additionally, GSK-3β and Akt were investigated as upstream regulators of tau phosphorylation. The current study did not identify changes to GSK-3β or Akt phosphorylation at Ser9 or Ser473, respectively.

5.3 Future Directions

The findings presented herein represent the first step in the characterization of the novel ACHI model. The positive results identified by the current study warrant further utilization of this model to elucidate the underlying pathophysiology of rmTBI in the juvenile rodent. Future directions involve, firstly, further characterization of the model by varying the number of impacts delivered, the duration between impacts, and the examination of injury without the use of the helmet. The present study did not investigate learning and memory following brain injury. This is an important future direction considering the prevalence of learning and memory impairment following concussion. The MWM is a great candidate for further analysis.

Additionally, the Barnes Maze and Novel Object Recognition test are other potential options for evaluating cognitive deficits following rmTBI with the ACHI model. The findings herein could be strengthened with the comparison to mTBI animals that sustain only one injury prior to behavioural testing. Moreover, the ACHI model provides the opportunity to make a direct comparison between anaesthesia and awake outcomes post-injury.

For initial investigations with this novel model, a post-injury time-point of seven days, PID 7, was chosen for the investigation of tau phosphorylation. This time-point was chosen based on previous literature and for practical reasons. Reviewing the literature presented in **Table 1,** alterations to tau following brain trauma can occur as early as 1-6 hours and may persist as long as six months. PID 7 represents a relatively central time-point that is neither acute, nor chronic. Practically speaking, tissue was collected beyond the window of the behavioural testing completed herein. Future directions include investigation of an acute time-point, such as one hour or 24 hours post injury, as well as the inclusion of a more chronic time-point such as one month post. Moreover, tau contains 80 serine/threonine sites, such that examination of additional epitopes may reveal interesting results. GSK-3β is one of the primary tau kinases, although ERK1/2 and cdk5 may represent other potential tau kinases of interest. The biochemical analysis performed in the present study, and in future research would be complemented with immunohistochemistry to provide spatial information.

5.4 Summary and Conclusions

The current study demonstrates that rmTBI delivered with the novel ACHI model produces acute neurological impairment and neurobehavioural sequalae similar to that of clinical cases. NSS testing demonstrated acute impairment to consciousness following each injury, deficits in gross motor function, and that repeat injuries resulted in worse outcomes than only a

single injury. Behavioural tests detected anxiety-like behaviour and deficits to motor coordination that were transient post-injury. These functional disturbances were identified in the absence of alterations to phosphorylation of tau protein or related kinases.

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Appendix

Appendix A. rmTBI decreases total tau in the ipsilateral CA

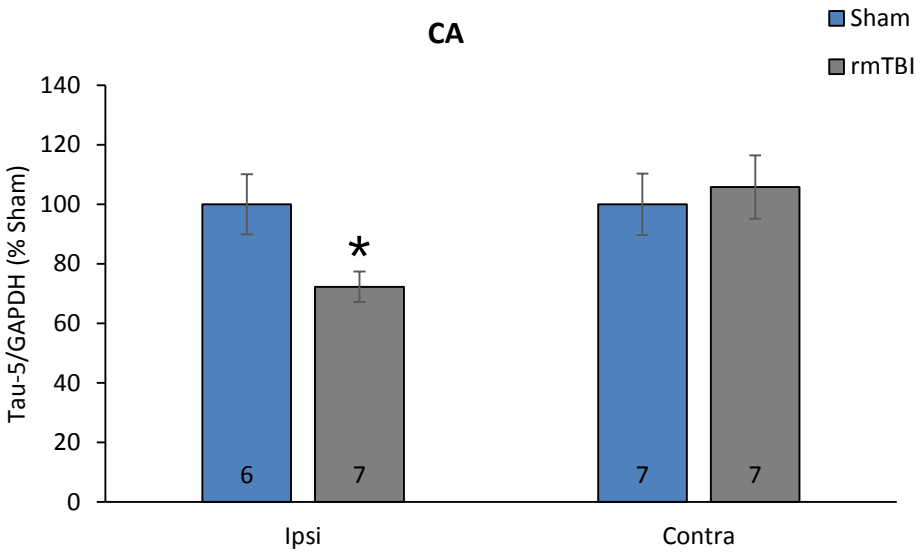

Figure I. rmTBI reduces total tau protein levels in the CA. Western blot analysis of Tau-5 in juvenile sham and rmTBI rats. Relative amounts of protein was calculated as a ratio of Tau-5 to GAPDH, and presented as a percentage of sham. Changes in Tau-5 were observed only in the ipsilateral (ipsi) CA. Data is expressed as Mean ± SEM. Number of samples are denoted in the respective bars. * $p < 0.05$.

Appendix B. Open-field test literature review

Author	Year	Animals	Injury model	Setup	Open field
Jones et al.	2008	Male Wistar rats (8-12 weeks old)	Lateral FPI	Timepoints: 1, 3, and 6 months after injury	1 m diamtere circular arena, 20 cm walls. INNER CIRCLE (66 cm diameter) -- **10 min trials**
Results:	*OF: time in center + number of center entries decreased in TBI animals, significant at 1 and 3 mo post. No difference in distance travelled*				
Pandey et al	2009	Male Wistar rats (250-300g)	Weight drop (400g) // central impact onto metal disc //exposed skull // cushioning foam bed	Open field: PID 25	90 cm circulular arena - **5 min trial** - Measured distance traveled, number of rearing episodes, number of fecal droppings (MORNING)
Results:	OF: TBI animals showed increased movement, rearing and defecation				
Kwon et al.	2011	Male Sprague-Dawley rats (245-265g)	Blast TBI; whole body blast overpressure	24h, 1 mo, 2 mo post injury. OF -> EPM -> BM	40 x 40 x 30 cm clear Plexiglas arena - **60 min testing period**; MEASURE: horizontal activity, time spent in the margins, time spent in the center
Results:	*OF: stress injured animals spent more time in periphery and less in center @ 24h PI*				
Shultz et al.	2011	Male Long-Evans rats (Adult)	3mm craniotomy; Later fluid percussion injury	DAY 2: in OF	Measure locomotor behaviour: 90 cm diameter, 40 cm walls -- **10 min trial**; MEASURE: total distance traveled (individual)
Results:	*OF: no differences*				
Kane et al.	2012	C57BL/6J	Weight drop + flip 5 impacts - 1/d for 5 days	Test 5 or 30 days post injury	43 cm x 42 cm x 42 cm transparent plastic cages; MEASURE: total activity as sum of all beam breaks in both horizontal and vertical planes - - 30 min trial
Results:	*Locomotor activity increased at 5 days post, no difference by 30 days post*				
Shultz et al.	2012	Adult male Long-Evans	LFPI (1.0-1.5 atm); 1, 4, 5 impacts	PID 1	LOCOMOTOR AND SOCIAL BEHAVIOUR // Circular arena; 90 cm diameter, 40 cm high walls -- **10 min trials**
Results:	*OF: no difference*				
Washington et al.	2012	Adult male C57BL/6J mice (3mo)	CCI: 5.25 m/s -- 1.5 mm depth (mild); 2.0mm (mod); 2.5 mm (severe)	OF: PID 21	Gross motor ability and exploratory behavior assessed 21 days post injury using OF; 3 FT diameter white novel arena -- 5 min trial -- MEASURE: speed, total distance travelled, time spent in center vs time spent outer area
Results:	*No differences in OF*				

Yu et al.	2012	Male C57BL/6 (8 weeks old)	CCI - left parietal /stereotax/4 mm diamter craniotomy	Test 10 days post	40 cm x 40 cm square arena; MEASURE: total distance traveled, time spent in center zone -- 10 min trials (center defined as 20cm x 20cm inner square)
Results:	*TBI mice traveled longer distance (hyperexcitability). TBI mice spent less time in center (anxiety)*				
Almeida-Suhett et al	2014	Male Sprague-Dawley rats (5-6 weeks old)	CCI: stereotax, craniotomoy - 3.5 m/s - 2 mm deformation	Animals tested 2 days before and 1, 7, and 30 days PI	40x40x20 cm clear Plexiglas arena - **20 min trials** - MEASURE: distance traveled, total time moving, vertical activity, time spent in center… Anxiety = ratio of time spent in center/total movement time
Results:	*TBI caused increased anxiety (spent less time in center of open field) 7 days after CCI which persisted to 30 days post. No difference in vertical activity, time moving, nor total distance*				
Cheng et al.	2014	Male and female C57Bl/6J mice at 4-6 months	Frontal impact (2 hit vs 1 hit); CCI -- craniotomy, stereotax, 3m/s, 1.5 mm depth	2-4 weeks post injury	Clear plastic chamber (41 x 41 x 30 cm) -- 5 min trials -- MEASURE: total movements in center and the periphery + rearing
Results:	*No significant differences found 2-4 weeks after injury in OF between 2X and sham mice*				
Ghadiri et al.	2014	Male Wistar rats (230-300g)	Modified weight drop (pendulum); craniotomy; stereotax	Timepoints: Before TBI, 24 h, 48 h, 7d, 14d post injury	50 x 50 cm squared quadrant -- **5 min trials**; MEASURE: grooming time, number of rearing, locomotion
Results:	*Locomtion decreased at 24h post, returned to pre-injury levels after 48h. TBI rats showed significant decrease in rearing activity at 24 and 48h post*				
Illiff et al.,	2014	Male C57BL/6 (8-12 weeks old)	"Hit and Run" TBI	1x per week for 4 weeks	10 min trial // motor activity
Results:	*No difference*				
Mannix et al.	2014	Male C57BL/6 (8-12 weeks old)	Closed head weight drop + flip; INJURED: 7 injuries	OF: Day 15, 3 months post injury	45 cm diameter circle, 20 cm walls - divided in 3 concentric circle sections. Inner circle 20 cm diameter, "neutral ring" 20 cm, outer 40 cm -- **10 min trials** --
Results:	*PID 15: no differences // PID 90: injured rats spent less time in outer zone, travelled greater distance*				
Arain et al.	2015	Male Sprague-Dawley rats (70-75 days old)	Moderate severity CCI: 4m/s directly to brain	OF: PID13, Sacrifice PID31	90 x 90 x 40cm square - **10 MIN TRIALS**; MEASURE: total distance traveled, percent time in outer area, average velocity
Results:	*No locomotor deficits (OF + EPM)*				
Johnstone et al	2015	Male Sprague-Dawley (12 weeks old)	LPFI	12 week post injury	Locomotion and axneity-like behaviour // 100cm diameter, 20cm walls, centre 66 cmTRIAL: **10 mins**

Results:	*OF: decreased time in centre*				
Mychasiuk et al.	2015	Male and Female Sprague Dawley rats (P30)	Modified weight drop (Kane et al., 2012) Injury on P30, P60, or P30 and P60 (4 groups)	Open field 2 days post	Circular field: 100 cm diameter - **10 min testing period**; MEASURE: distance travelled, speed of travel
Results:	*OF: P30 injuried animals recovered by PID62, animals injured on P60 or P30+P60 displayed reduced distance covered*				
Shultz et al.	2015	Long-Evans rats (12 weeks old)	Lateral FPI (4 atm)	12 weeks post injury	Circular arena; 100 cm diameter, 20 cm high walls. INNER CIRCLE (66 cm) -- **10 min trials**
Results:	*OF: no significant effects*				
Shultz et al.	2016	Adult male Long-Evans (12 weeks old)	mFPI (1-1.5 atm)// 3 injuries; 5 day inter injury period	3 months post injury	Circular arena; 100 cm diameter, 20 cm high walls. INNER CIRCLE (50 cm) -- **10 min trials**
Results:	*OF: no difference*				

Appendix C. Elevated-Plus Maze literature review

Author	Year	Animals	Injury model	Setup	Elevated plus maze
Jones et al.	2008	Male Wistar rats (8-12 weeks old)	Lateral FPI	Timepoints: 1, 3, and 6 months after injury	EPM: **10 min trials**
Results:	*EPM: TBI animals spent less time in open arms, less entries to open arms, significant at 1 and 3 months*				
Pandey et al.	2009	Male Wistar rats (250-300g)	Weight drop (400g) // central impact onto metal disc //exposed skull // cushioning foam bed	EPM: PID 25	Two open (50cm x 10cm) arms, two closed arms (50cm x 10cm x 30cm). 10cm x 10cm central platform. **5 MIN TRIALS**
Results:	*EPM: TBI rats showed increase % of entries and increase % of total time in open arms*				
Kwon et al.	2011	Male Sprague-Dawley rats (245-265g)	Blast TBI; whole body blast overpressure	24h, 1 mo, 2 mo post injury. OF -> EPM -> BM	1 m above ground, arms 50 cm long x 10 cm wide, 40 cm high walls on closed arms. **5 MIN trials** to explore
Results:	*EPM: stress injured animals traveled less distance 48h Pi; injured animals spent less time in open arms and more time in closed arms @ 48h PI – persisted to 1 mo, no differences by 2 mo*				

Shultz et al.	2011	Male Long-Evans rats (Adult)	3mm craniotomy; Later fluid percussion injury	DAY 1: EPM	55 cm long arms x 12 cm wide, 50 cm above the floor; CLOSED ARMS: 18 in high walls**5 min trial**
Results:	*EPM: SR injured rats exhibited increased time spent in open arms compared to shams. No difference in closed arm entries. LR injured rats showed no differences from sham*				
Zohar et al.	2011	Male ICR mice	Modified weight drop (80 cm; 20, 25, 30 g)	7, 30, 60, 90 days post	Assess anxiety // 30x5x15 arms; elevated 60 cm // 5 MIN TRIALs
Results:	*No differences*				
Shultz et al.	2012	Adult male Long-Evans	LFPI (1.0-1.5 atm); 1, 4, 5 impacts	DAY 1. EPM, then OF	EPM: 50cm x 12 cm open; 50 cm x 12 cm x 50 cm closed. **5 min trials**
Results:	*EPM: no difference*				
Washington et al.	2012	Adult male C57BL/6J mice (3mo)	CCI: 5.25 m/s – 1.5 mm depth (mild); 2.0mm (mod); 2.5 mm (severe)	EPM PID 21	15 inches above ground, 26 in long arms
Results:	*EPM: no difference in distance travelled, similar total arm entries = same exploratory behaviour; significant effect of injury on time spent in open arms == reduced anxiety in CCI mice*				
Johnson et al.	2013	Adult Sprague-Dawley rats (4 months)	Frontal CCI (2.25 m/s, 3mm depth, 50 ms contact)	PID 7	EPM: 102cm x 10cm open; 100 x 10 x 30 cm closed. **5 min trials**
Results:	*Increased time in open arm; risk taking behaviour*				
Cheng et al.	2014	Male and female C57Bl/6J mice at 4-6 months	Frontal impact (2 hit vs 1 hit); CCI – craniotomy, stereotax, 3m/s, 1.5 mm depth	2-4 weeks post injury	Elevated 63 cm from ground – 10 min trial – MEASURE: locomotor activity and percent of time spent in open versus closed arms
Results:	*No significant differences found 2-4 weeks after injury in OF or EPM between 2X and sham mice*				
Logsdon et al.	2014	Adult male 124prague-dawley rats (300-350g)	Blast (mild ~15 psi)	PID 7	EPM: 50cm x 10 cm open; 50 cm x 10 cm x 30 cm closed. **5 min trials**
Results:	*bTBI animals spent significantly more time in open arm*				
Mannix et al.	2014	Male C57BL/6 (8-12 weeks old)	Closed head weight drop + flip; INJURED: 7 injuries	EPM: Day 17, 3 mo post injury	2 open, 2 closed arms (30 x 5 cm); raised 85 cm. SETUP: place mouse in center facing closed arm – 5 min trial

Results:	*PID 15: no differences // PID 90: injured rats spent less time in outer zone, travelled greater distance*				
Mouzon et al.	2014	Male C57BL/6J (9-15 months old)	Sham/1 hit/repeat sham/ rmTBI (5 hits over 10 days)	12 Months post injury	Elevated 50cm; arms: 55cm long x 4cm wide, 25cm high walls in closed arms; 5 min trial
Results:	*12 mo post injury: shams spent less time in open arm thatn s-mTBI or r-mTBI*				
Petraglia et al.	2014	Adult male C57BL/6J (12 weeks)	Awake CCI: sham, single, or repeat (42 impacts)	14 d, 1 mo, 6 mo post impact	EPM: 35 x 5 cm open arms/ 35 x 5 x 15 cm closed arms/platform 5x5 cm/ 60 cm above floor; **5 MIN trials**
Results:	*2 weeks post: mTBI mice exhibited anxiety-like before (less time in open); no difference between single and sham at 1 and 6 months; at 1 mo rmTBI mice spent more time in open arm = increased risk taking*				
Arain et al.	2015	Male Sprague-Dawley rats (70-75 days old)	Moderate severity CCI: 4m/s directly to brain	EPM PID15, Sacrifice PID31	60cm long x 20cm wide, 60cm off the ground - **10 MIN TRIALS**
Results:	*no anxiety-associated behaviours (EPM)*				
Johnstone et al.	2015	Male Sprague-Dawley (12 weeks old)	LPFI	12 week post injury	EPM: 50cm x 12 cm open; 50 cm x 12 cm x 50 cm closed. **5 min trials**
Results:	*EPM: decreased time in open arms*				
Mychasiuk et al.	2015	Male and Female Sprague Dawley rats (P30)	Modified weight drop (Kane et al., 2012) Injury on P30, P60, or P30 and P60 (4 groups)	EPM 3 days post	55 cm above ground; **10 min trial**
Results:	*EPM: P30 injuried, no difference. P60 and P30 +P60 injured animals spent less time in open arms than shams*				
Nichols et al.	2016	Adult male C57BL/6 mice (3 months)	Modified Maramou weight drop // Single or repeat mTBI (3 impacts, 24h inter-injury interval)	EPM: PID 9	40 cm high, 30 x 5 cm arms, closed with 15.25 cm armsTRIALS: 4 minutes
Results:	*No difference from single or repeat injury*				
Shultz et al.	2015	Long-Evans rats (12 weeks old)	Lateral FPI (4 atm)	12 weeks post injury	EPM: **5 min trials**
Results:	*EPM: no difference between SHAM and TBI, significant effect of sodium selenate*				

Shultz et al.	2016	Adult male Long-Evans (12 weeks old)	mFPI (1-1.5 atm)// 3 injuries; 5 day inter injury period	3 months post injury	EPM: 50cm x 10 cm open; 50 cm x 10 cm x 30 cm closed. **5 min trials**
Results:	*EPM: results not stated!*				
Turner et al.	2015	Adult male rats (300-350g)	Blast (~50 psi)	PID 7	
Results:	*Single blast and repeat blast animals spent more time in open arm compared to sham*				
Mannix et al.	2017	Male C57BL/6 (ADULT: 16 weeks; JUVENILE: 5 weeks)	Modified weight drop (7 hits/9 days or 4 hits/4 days)	PID 21, 3 months post	EPM used to test impulsivity // 5 min trials
Results:	*7 hits/9 days: increased time in open at PID 21 // 4 hits in 4 days: no differences*				

Appendix D. Rota-Rod literature review

Author	Year	Animals	Injury model	Setup	Rota-Rod
Longhi et al.	2010	Male C57BL/6 (6-8 weeks old)	Modified CCI; stereotax; left parietal -- 4.8 - 5.0 m/s; 3 mm depth	Single impact; 2nd injury at 3, 5, or 7 days after MWM testing on day of last injury; 24h after rotarod testing	4 days post injury, rotarod used to evaluate motor function; 1 acclimization session; 4 trials at 5 minute intervals; MEASURE: latency on the rod - START: 1 cm/s and accelerating at 1.75 rpm/s
Results:	*All injured animals showed shorter latencies than SHAM. Animals receiving 2 concussions within 3 days showed the greater deficits. A second concussion at 5 or 7 days after the first resulted in the same deficits as only a single injury*				
Kane et al.	2012	C57BL/6J	Weight drop + flip 5 impacts - 1/d for 5 days	24h or 7 days post injury using the acceleration Rotarod from Ugo Basile	Accelerate from 4 to 40 rpm over 5 mins. Max time 300s; MEASURE: time spent on apparatus
Results:	*Performance decreased after 4x hits and 1 D post injury, no significant change after 5x hits or 10x hits and waiting 10 days*				
Yu et al.	2012	Male C57BL/6 (8 weeks old)	CCI - left parietal /stereotax/4 mm diamter craniotomy	4 days training prior. Test 1, 3, 7 days post-surgery: 4 trials, 5 mins minimum between surgery	UGO BASILE Rotarod - Accelerate from 4 to 40 rpm in 4 mins; maintained at 40 rpm for 1 min; MEASURE: latency to fall or cling for two full rotations
Results:	*Significant deficits at 1 and 3 days, recovered at 7 days after injury*				

Yan et al.	2013	Male Sprague-Dawley rats (12 weeks old)	Weight drop (6.15m/s) - exposed skull	2 training trials, week before. Tested 24h post injury	Ratek, VIC rotarod test -- Rotating speed increased in increments of 1.5 rpm every 3s; RECORD: highest speed before falling off
Results:	*2 days prior to injury, 24h post injury = No significant differences between groups before injury. Dose dependent effect of increasing TBI velocites on performance*				
Yang et al.	2013	Male C57/BL6 (8-10 weeks old)	Weight drop (closed head; 1.5cm 400-500g)	Training for 5 days. Injury on DAY 6, Testing resumed on DAY 7 (24 hr post injury) for another 5 days	IITC Life Science Rotarod device -- TRIAL 1: 5 rpm accelerate to 24 rpm over 90s. TRIAL 2: 5 rpm start accelerate to 36 rpm over 180s
Results:	*Over 5 d training; observed increased skill (learning curve) = increased time on rotarod. Shams continued to improve, whereas there was a significant decrease*				
Illiff et al.	2014	Male C57BL/6 (8-12 weeks old)	"Hit and Run" TBI	2 days pre-training. Test: 7, 14, 21, 28 days post	Start speed: 10 rpm, accelerated 1 rpm/s for a max of 6 min. 3 sessions per day
Results:	*No effect*				
Luo et al.	2014	FVB/N reporter mice expressing GFAP and WT C57BL/6J mice (2-3 mo)	Modified CCI - closed head	3 months post	Ugo Basile Rota-Rod; SETUP: accelerate from 5 to 30 rpm during a test period of 5 min. Mice were tested 3 times, 20 mins between trials; MEASURE: latency to fall off
Results:	*No difference between sham, 1 hit, or 3 hits*				
Mannix et al.	2014	Male C57BL/6J (2-3 months old)	Modified weight drop (7 hits/9 days)	PID 1-3; 30 days post	START: 4 rpm for 10 s; ACC: 0.1 rpm/s / 4 trials
Results:	*Decreased performance PID1-3 + decreased performance 3 mo post*				
Mouzon et al.	2014	Male C57BL/6J (9-15 months old)	Sham/1 hit/repeat sham/ rmTBI (5 hits over 10 days)	DAY 1: pretraining // 6 months post single or repeat mTBI	Ugo Basile Rota-Rod; SETUP: accelerate from 5 - 50 rpm over 5 minute period; TRIALS: 3 per day, intertrial interval of 5 min
Results:	*No differences = "transient nature of motor deficits*				
Maegele et al.	2015	Male Sprague-Dawley rats (300-350g)	Lateral fluid percussion	Tests 24 h, 7, 15, 90 days post injury	TRIALS: 3 trials, minimum 5 mins rest between trials -- Increasing speed of 0 to 30 rpm within 60s;
Results:	*Significant impairment in Time, Distance and Speed on rota-rod performance that remained at PID 90*				
Mannix et al.	2017	Male C57BL/6 (ADULT: 16 weeks;	Modified weight drop (7 hits/9 days or 4 hits/4 days)	1 Day training. TEST: PID 1-3; 30 days post	START: 4 rpm for 10 s; ACC: 0.1 rpm/s / 4 trials

		JUVENILE: 5 weeks)			
Results:	*7 hits/ 9 days: decreased performance at PID 1-3 + 3 months // 4 hits/day: no deficits*				

Appendix E. Open-field test literature review

Author	Year	Animals	Injury model	Setup	Forced Swim Test
Milman et al.	2005	Male ICR mice	Weight drop (30g from 80cm)	7d, 1 mo, 2 mo, 3 mo	Depressive-like state was assessed. TRIALS: 6 min // Clear plexiglas cylinder (height 25 cm, diameter 10 cm), 6 cm water. MEASURE: last 4 mins of trial - time spend
Results:	*Significant difference at all time-points*				
Taylor et al.	2006	Adult male Sprague-Dawley rats (60 - 70 days old)	CCI + stereotax, midline incision, craniotomy // TBI = 2.75 m/s; 2.00 mm depth	8 weeks post	Plexiglas cylinder: 45 cm high x 25 cm diameter; 35 cm water @ 25˚C; TRIAL: 5 min test phase
Results:	*No differences in immobility; TBI rats spent less total time swimming*				
Jones et al.	2008	Male Wistar rats (8-12 weeks old)	Lateral FPI	6 months post injury	Used to measure depression-like behavioural despair // Clear Perspex cylinder (30cm diameter x 40cm height), filled with 30cm water @ 25˚C // MEASURE: time immobile, time spent climbing, time swimming. Only score behaviours that persisted for more than 2 sec.
Results:	*No difference*				
Shultz et al.	2011	Male Long-Evans rats (Adult)	3mm craniotomy; Later fluid percussion injury	FST training on Day 3, on Day 4 of behavioural testing // 24h or 4 w post injury	Depression-like behaviours were assessed using the FST. TRIALS: 5 minutes // Clear glass cylinder: 20 cm diamter x 30 cm deep water @ 25˚C //MEASURE: time spent immobile, time spent climbing, time spent swimming
Results:	*No difference*				
Zohar et al.	2011	Male ICR mice	Modified weight drop (80 cm; 20, 25, 30 g)	7, 30, 60, 90 days post	25 cm, 6cm of water // 6 MIN TRIAL, measure last 4 min

Results:	*All time-points post injury showed increased immobility*				
Shultz et al.	2012	Male Long-Evans (250-300g)	Repeat mLFP (craniotomy; mild: 1.0-1.5 atm) // 1, 3, or 5 hits	PID 3 - 15 min training session; PID 4 - 5 min test session	Depressive-like behaviors assessed using FST. TRIALS: 5 mins (30 cm water)
Results:	*24 hours post; no differences 5 hit, 8 weeks post - increased time immobile*				
Washington et al.	2012	Adult male C57BL/6J mice (3mo)	CCI: 5.25 m/s -- 1.5 mm depth (mild); 2.0mm (mod); 2.5 mm (severe)	OF, EPM same day, 2 hr break between: PID 21	PID 21 using the Porsolt mouse FST; 2 L beaker filled with 7-8 cm of water (25˚C) -- 6 MIN TRIALs -- MEASURE: last 4 mins of the test
Results:	*CCI mice display despressive-like behaviour (all Sev.). Immobility time significantly increased in CCI mice compared to sham*				
Cheng et al.	2014	Male and female C57Bl/6J mice at 4-6 months	Frontal impact (2 hit vs 1 hit); CCI -- craniotomy, stereotax, 3m/s, 1.5 mm depth	5 day PI and 6 months post injury	Clear polycarbonate cylinder: 31 cm diameter x 76 cm filled to 48 cm with RT tap water -- 6 min trials -- As described by Petraglia et al 2014
Results:	*No differences between 2X mice and sham at either timepoint*				
Petraglia et al.	2014	Adult male C57BL/6 mice (3 months)	Awake model/ CCI	1 month post injury	Open glass cylinder (12 cm diameter, 24 cm high, 16 cm fresh tap water @ 23-25˚C -- 6 min trial -- last 4 mins used for analysis
Results:	*Significant increase in duration of immobility in rmTBI group compared to single hit and sham // no difference between mTBI and Sham*				
Mychasiuk et al.	2015	Male and Female Sprague Dawley rats (P30)	Modified weight drop (Kane et al., 2012) Injury on P30, P60, or P30 and P60 (4 groups)	FST on P75	Cylindrical tank: 60 cm high x 30 cm diamter filled with enough water so the rat's tail couldn't touch the bottom. 22˚C // TRIAL: 7 mins. // Between all trials the tank was emptied, cleaned with Virkon and refilled// MEASURE: amount of time the rat spent immobile
Results:	*No differences observed when injury delivered on P30 vs P60 or P30 + P60 compared to SHAM*				
Shultz et al.	2015	Long-Evans rats (12 weeks old)	Lateral FPI	12 weeks post injury	Depression-like behaviours were assessed using the FST. TRIALS: 5 minutes // Clear glass cylinder: 20 cm diamter x 30 cm deep water @ 25˚C //MEASURE: time spent immobile, time spent climbing, time spent swimming
Results:	*No effect*				
Nichols et al.	2016	Adult male C57BL/6 mice (3 months)	Modified Maramou weight drop // Single or repeat mTBI (3 impacts, 24h inter-injury interval)	FST on PID 10	4 L beaker: 15.25 cm diameter x 25.5 cm high filled with 2500 ml water @ 28˚C -- 6 minute trial -- LAST 4 MINS evaluated; MEASURE: immobility duration

Results:	*No difference from single or repeat injury*				
Tan et al.	2016	Adult male Long-Evans (12 weeks old)	mFPI (1-1.5 atm)// 3 injuries; 5 day inter injury period	3 months post injury	Depression-like behaviours were assessed using the FST. TRIALS: 5 minutes // Clear glass cylinder: 20 cm diamter x 30 cm deep water @ 25˚C //MEASURE: time spent immobile, time spent climbing, time spent swimming
Results:	*3mFPI caused significant increase in time immobile*				