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From Trauma To Neurodegeneration: A One-Year Time Progression Of Functional Impairments And Its Associated Neuropathological Link Following Varied Severity Of Experimental Diffuse Traumatic Brain Injury

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Declaration

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Publications and Presentations

Publications

i. Reviews

Corrigan F, **Arulsamy A**, Teng J, Collins-Praino L, Pumping the brakes: Neurotrophic factors for the prevention of dementia following traumatic brain injury, *Journal of Neurotrauma*, 34(5); 971-986, 2017

ii. Original Research Papers

Corrigan F, **Arulsamy A**, Collins-Praino L, Holmes J, Vink R, Toll like receptor 4 activation can be either detrimental or beneficial following mild repetitive traumatic brain injury depending on timing of activation, *Brain, Behavior, and Immunity*, 64(3); 124-139, 2017

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Arulsamy A, Corrigan F, Collins-Praino L, Cognitive and neuropsychiatric impairments vary as a function of injury severity at 12 months post-experimental diffuse traumatic brain injury: Implications for dementia development, *Behavioural Brain Research*, 365; 66-76, 2019

Presentations

Arulsamy A., Teng J., Colton, H., Corrigan F., and Collins-Praino L.E, **10th Florey International Postgraduate Research Conference,** Poster Presentation, September 2016, Adelaide, Australia

Arulsamy A., Teng J., Colton, H., Corrigan F., and Collins-Praino L.E, **36th Australasian Neuroscience Conference 2016 (ANS 2016)**, Poster Presentation, December 2016, Hobart, Australia

Arulsamy A., Teng J., Colton, H., Corrigan F., and Collins-Praino L.E, **8th Australian Neurotrauma Symposium,** Poster Presentation, December 2016, Hobart, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **7th Australasian Cognitive Neuroscience Society Conference 2017 (ACNS 2017)**, e-Poster Presentation, November 2017, Adelaide, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **37th Australasian Neuroscience Conference 2017 (ANS 2017)**, Poster Presentation, December 2017, Sydney, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **28th HUPO Brain Proteome Project Workshop,** Oral Presentation, May 2018, Adelaide, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **ASMR SA Annual Scientific Meeting,** Oral Presentation, June 2018, Adelaide, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **3 Minute Thesis Competition (3MT)-Faculty Heat,** Oral Presentation, August 2018, Adelaide, Australia

Arulsamy A., Corrigan F., and Collins-Praino L.E, **12th Florey International Postgraduate Research Conference,** Poster Presentation, September 2018, Adelaide, Australia

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~ Colin Powell.

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Abbreviations

5C-SRT – 5 Choice Serial Reaction Time

5C-CPT – 5 Choice Continuous Performance Task

AD – Alzheimer's disease

ALS – Amyotrophic Lateral Sclerosis

ANOVA – Analysis of variance

BM – barnes maze

CCI – controlled cortical impact

CD68 – Cluster of Differentiation 68

CHIMERA – closed head impact model of engineered rotational acceleration

CTE – Chronic traumatic encephalopathy

DAI – Diffuse axonal injury

EPM – elevated plus maze

FPI – fluid percussion injury

FST – forced swim test

FTD – fronto-temporal dementia

GCS – Glasgow coma scale

GFAP – Glial fibrillary acidic protein

HR – hazard ratio

Iba1 – Ionized calcium binding adaptor molecule 1

IHC – Immunohistochemistry

ITI – inter trial interval

LOC – loss of consciousness

MBP- myelin basic protein

MCI – mild cognitive impairment

MND – motor neurone disease

mTBI – single mild traumatic brain injury

msTBI – moderate to severe traumatic brain injury

MWM – morris water maze

NeuN – Neuronal nuclei

NF-L / NF-H – neurofilament light/ heavy

OFT – open field test

OR – odds ratio

PBS – Phosphate buffered saline

PD – Parkinson’s disease

PFC – prefrontal cortex

PSD-95 – Post-synaptic density protein 95

rmTBI – repetitive mild traumatic brain injury

SEM – Standard error of the mean

SN – substantia nigra

SOD-1 – Superoxide dismutase 1

TBI – Traumatic Brain Injury

TDP-43 - TAR DNA-binding protein 43

TBST – Tris buffered saline with tween

WB – Western Blot

Abstract

Traumatic brain injury (TBI) is seen as more than just a static insult but an injury with a disease like progression, in which it is deemed as a risk factor for neurodegenerative diseases later in life. Clinical studies have shown a severity dependent relationship between TBI and neurodegenerative diseases. However, there is still a lack of understanding on the timely progression of experimental diffuse TBI into long-term impairments as well as the brain mechanism accounting for these deficits. Furthermore, many preclinical studies have not investigated this progression in relation to the severity of the initiating insult. Therefore, this study investigated the timely change in functional outcomes through a behavioral battery assessing general motor activity, anxiety, depression and cognition as well as its associated neuropathology up to 12 months post injury following varied TBI severities.

To investigate this aim, this study first use male Sprague Dawley rats (10-12 weeks) which were subjected to either sham surgery or Marmarou's impact acceleration model of moderate to severe diffuse TBI, in determining the functional and neuropathological changes at early sub-chronic stages (1 and 3 months) post injury. Since the moderate to severe TBI animals in this study showed persistent depressive-like impairments up to 3 months coupled with subtle cognitive flexibility deficits (supported by prefrontal cortex neuropathology), the study continued in determining the progression of these functional impairments at later chronic stage (12 months) post injury while concurrently determine if injury severity (single mild, repetitive mild (3 mild diffuse injury at 5 day intervals) and moderate to severe TBI) may be a factor influencing them. Moderate to severe TBI continue to display significant cognitive flexibility impairments at 12 months that were not present in other severity groups when compared to shams. However, no other functional deficits were present at 12 months post injury regardless of injury severity. Thus, to explore further the cognitive deficits, the study delved deeper into determining the injury severity effect on the evolution of executive function using the touchscreen cognitive paradigm up to 12 months post injury. The effect of age, but not

injury severity on executive dysfunction was revealed. Lastly, this study sought out to determine the associated neuropathology to the functional impairments seen at 12 months post injury through molecular analysis of neurodegenerative disease related brain areas and spinal cord regions after different severities of TBI. Cytoplasmic mis-localization of TDP-43 proteins in the cervical spinal region with abnormal changes in NeuN and phosphorylated-TDP-43 levels were found in motor cortex and spinal regions of the single and repetitive mild TBI animals only at 12 months post injury. No other neuropathology was seen in the other brain regions regardless of injury severity.

Thus, overall this thesis suggest that injury severity and age play as important factors in predicting long term functional outcomes post injury, with cognitive impairments in moderate to severe TBI suggesting implication towards later dementia development, while mild diffuse TBI may have higher implications towards motor neurone diseases based on the neuropathological evidence. This thesis advocates the significance of understanding the temporal profile of functional deficits and accompanying neuropathological changes that occur in the months and years following TBI which is critical for improving the predictability of neurodegenerative disease risk following TBI.

Chapter 1

Nature and severity of TBI influences long-term outcomes post injury: possible relationship to neurodegenerative disease

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Nature and severity of TBI influences long term outcomes post injury: possible relationship to neurodegenerative disease

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Abstract:

Traumatic brain injury (TBI) is a huge economic burden worldwide due to its high rate of mortality and morbidity. Additionally, it is also deemed a significant risk factor for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Despite the growing body of evidence linking TBI with the later emergence of neurodegenerative disease, it is still unclear how the severity of the initial injury may affect this risk. Some recent work has hypothesised that different severities of injury may be linked with increased risk of different neurodegenerative disease, with repetitive mild injuries associated with chronic traumatic encephalopathy (CTE) and moderate/severe injuries leading to Alzheimer's disease. The current review evaluates the evidence for this claim, by critically assessing the clinical and pre-clinical body of research to date, relating to neurodegeneration as well as long-term behavioural outcomes post varied severities of TBI. While there is strong evidence on the TBI severity effect against later life impairments in later years, reflection of these studies in preclinical settings are scarce, majorly due to the limited number of late chronic (beyond 3 months) studies available. This number decreases even further when filtered for a varied TBI severity within a single cohort or for the more clinically-relevant diffuse TBI. Therefore, to shorten the gap between clinical and preclinical TBI research, particularly with neurodegeneration, understanding the severity-dependent late chronic behavioural outcomes post diffuse experimental TBI should be the focus of future preclinical studies.

1. Introduction

Traumatic brain injury (TBI) is a major cause of disability and mortality worldwide. The Centre of Disease Control and Prevention (CDC) stated that, in 2010 alone, there were approximately 2.5 million emergency department visits, hospitalisations and deaths due to TBI (Faul et al., 2010; Frieden et al., 2014). Although previous estimates have suggested that 10 million people a year are affected by a new TBI event (Hyder et al., 2007), a recent study by Feigin and colleagues (2013) argued that by extrapolating from estimates in a New Zealand population-based study, as many as 54-60 million people annually may be affected by a new TBI (Feigin et al., 2013). A 1993 review by McGregor *et al* estimated the acute care cost to be as high as $80,000 per moderate TBI case (McGregor and Pentland, 1997) while Ashley *et al* (1997) saw a projection towards $450, 000 of annual post-acute care cost (Ashley et al., 1997). In addition, in 2010, the economic cost from just disability and loss of productivity post TBI was set at $76.5 billion (Alali et al., 2015). This huge economic burden could be attributed to the fact that TBI may not be just a single event of insult (Masel and DeWitt, 2010a) but will lead into secondary insults which have been linked to the development of neurodegenerative outcomes chronically (Chen et al., 2007a; Faden and Loane, 2015; Gardner et al., 2015b; Nemetz et al., 1999b; Omalu et al., 2005; Plassman et al., 2000; Sundman et al., 2014b).

Although most patients may regain full functionality after injury, in some cases functional deficits, especially in terms of cognition, motor and neuropsychiatric function, may persist for years following injury (Dikmen et al., 1995; Vincent et al., 2014a). Furthermore, TBI has also been recognized as a significant risk factor for the later development of Alzheimer's disease (AD), Parkinson's disease (PD) and even Amyotrophic Lateral Sclerosis (ALS) despite, physical recovery from the injury decades earlier (Guo et al., 2000; Lye and Shores, 2000; Mortimer et al., 1991; Sivanandam and Thakur, 2012; Vincent et al., 2014a). Current studies indicate that the risk may be dependent on the initial severity of the TBI (Barnes et al., 2018; Chen et al., 2007a; Raj et al., 2017; Tolppanen et al., 2017). While the risk of Parkinson's disease may be indifferent to TBI severity with only slight elevations in risk with severity

increment (Bower et al., 2003; Gardner et al., 2015b), dementia and AD were found to be higher in moderate to severe TBI patients (Barnes et al., 2018; Nordstrom and Nordstrom, 2018; Raj et al., 2017; Tolppanen et al., 2017), but the risk of neurodegenerative diseases like ALS and chronic traumatic encephalopathy on the other hand may be more associated with repetitive mild TBI (Chen et al., 2007a; McKee et al., 2013b).

In the current study, we will review the clinical and pre-clinical findings associated with the different severities (mild, moderate or severe) or nature (diffuse, focal) of TBI and their link to either neurodegeneration or its accompanying functional outcomes (cognition and motor performance).

2. Brief overview of traumatic brain injury

TBI is a form of acquired brain injury where an external force to the head causes a direct or indirect injury to the brain (Finnie and Blumbergs, 2002). TBI can be classified by type of injury as either an open head injury (penetration) or a closed head injury (without penetration of the skull) (Smith et al., 2003). Closed head injury can result in different types of TBI, either a diffuse or focal brain injury, depending on the force and direction of the impact (Blumbergs, 1997). A diffuse TBI, such as is commonly seen in road accident related TBI cases (Yattoo and Tabish, 2008), is the result of an act of sudden acceleration, deceleration or rotational force towards the head, causing certain parts of the brain to be compressed and axons to shear, thus resulting in diffuse axonal injury (Lv et al., 2014), oedema and vascular injury throughout the brain (Andriessen et al., 2010). Focal TBI, conversely, is caused by a direct force to the head, such as being hit with a blunt object or falling, resulting in damage to the soft gelatin-like brain on the inner side of the rigid skull in a coup-contrecoup manner (both site and opposite site of impact) and the formation of contusions and haemorrhages (Andriessen et al., 2010). At times, both a diffuse and focal injury can occur simultaneously at the point of impact (Andriessen et al., 2010).

In addition to categorising TBI based on the type/nature of injury, TBIs can also be classified based on their severity, mainly into three categories based on the immediate effects after the injury: mild, moderate or severe (Vincent et al., 2014a). The Glasgow Coma Scale (GCS), or a more recently proposed predictor known as Full Outline of UnResponsiveness (Fourgeaud et al., 2016), are used in hospitals to determine the severity of TBI in patients, with the score measuring the level of unconsciousness (Gajavelli et al., 2015) in the patient and predicting the possible outcome (Sadaka et al., 2012; Teasdale and Jennett, 1974) Mild TBI leads to unconsciousness of less than 30 minutes, GCS of 13 and above, headaches and swelling, while moderate and severe TBI may result in unconsciousness of more than 30 minutes, GCS of 9-12 for moderate and below 8 for severe, comatose state and even disability (Teasdale and Jennett, 1974). In addition to the severities associated with a single injury, there is also another subtype of mild TBI known as repetitive mild TBI. This type of TBI is usually seen in contact sports athletes. Despite protective gear such as helmets, this does not prevent rotational injuries, with evidence that even cumulative sub-concussive impacts may have long-term consequences.

3. Clinical studies

Over the past 20 years, there have been several population-based studies that have investigated the risk between a history of TBI and later onset of neurogenerative disease, either through a prospective study (since the onset of TBI) or a retrospective study (reported history of TBI in neurodegenerative patients). The odds ratio (OR) for dementia development following unspecified TBI or head trauma reported in these studies was between 1.57-2.8 (Barnes et al., 2014; Dams-O'Connor et al., 2013; Luukinen et al., 2005; van Duijn et al., 1992) with AD reporting an OR of 2.29 in males and 0.91 in females (Fleminger et al., 2003c) and the OR for frontotemporal dementia (FTD) following TBI was 1.67 (Deutsch et al., 2015b). Interestingly, some studies on AD showed no significant elevations in OR, suggesting that TBI may not be a risk factor, especially when coupled with loss of consciousness (LOC) (Crane et al., 2016;

Dams-O'Connor et al., 2013). Some of the strongest evidence for a link between TBI and neurodegenerative disease has emerged for PD, with an OR of between 1.3-3.56 (Crane et al., 2016; Harris et al., 2013; Lee et al., 2012; Taylor et al., 2016). In support of this, a recent meta-analysis performed by Huang and colleagues (2018), which includes the studies above, found a pooled OR of 1.93 (95% CI 1.47–2.55, $p < 0.001$) for dementia, 4.44 (95% CI 3.86–5.10, $p < 0.001$) for PD, and 2.97 (95% CI 1.35–6.53, $p < 0.001$) for TDP-43 associated disease (FTD and ALS) in individuals who had experienced a TBI (Huang et al., 2018).

In the general population, out of the three apolipoprotein alleles (E2, E3 and E4), the apolipoprotein E4 (APOE4) gene increases the OR for AD, PD and dementia (Guo et al., 2000; Jellinger et al., 2001; Luukinen et al., 2005), but Huang et al (2018) showed that there was no association between TBI and neurodegenerative disease when APOE4 genotype was accounted for, although this may be confounded due to only a handful of studies reporting APOE genotype (Huang et al., 2018). The APOE genotype is often discussed in studies investigating the link between TBI and dementia, as the presence of the APOE epsilon 4 gene is a significant contributor towards dementia development (Kivipelto et al., 2008). However, this genotype factor has not been investigated well in relation to a history of different severities of TBI. To our knowledge, only one study by Sundstrom (2007) concluded that those with both the APOE4 genotype and a history of mild TBI had a 5-fold risk increase of dementia, while no increase in risk existed in the absence of the genotype in a 5 year follow up population-based study (Sundström et al., 2007). Thus, whether this genotype also plays a role in more severe TBI should be investigated.

Interestingly, the OR tends to increase when the TBI is within 10 years from the onset of neurodegenerative disease (van Duijn et al., 1992) and when the age of TBI is younger, such as a study by Taylor *et al* (2016) suggesting that early life TBI increases the risk of PD, with OR increasing every 5 years with earlier TBI (Taylor et al., 2016). One study also stratified their data by gender and found a higher risk of neurodegenerative disease in men than in women

following a TBI, suggestive of potential neuroprotective effects of the hormone progesterone (van Duijn et al., 1992).

Nevertheless, many studies conducted to date have only measured the differences in OR between TBI with loss of consciousness and without, with the former having a higher OR (Crane et al., 2016; Dams-O'Connor et al., 2013). Most of these studies have not specified the severity (mild, moderate, severe) or nature of the TBI (whether single or repetitive), which could be confounding the OR. Thus, going forward, this review will limit its findings to severity and nature specified TBI studies and risk of neurodegenerative disease.

3.1 Dementia (general)

Dementia is defined as a loss of cognitive function, particularly memory, to the extent of interfering daily life activities (American Psychiatric Association, 2000). It is often regarded as an age-related disease. While some cases of dementia are associated with explicit hereditary/familial causes, environmental factors seem to play a critical role in the development of dementia in most individuals. TBI is the most researched environmental link to dementia to date. For the purposes of this review, we have separated studies into those that did not specific dementia type, those looking at AD risk and those investigating CTE, in order to better elucidate the relationship between TBI and specific forms of dementia.

As mentioned earlier, the risk of developing dementia is relatively high, up to 4-fold, when there's a history of TBI (Shively et al., 2012). However, could a simple bump to the head already increase this risk? Studies have found that a dose-response relationship exists between TBI and dementia; the higher the severity of TBI, the higher the risk factor, with single moderate-severe TBI displaying a hazard ratio (HR) between 1.35 to 3.77 (Barnes et al., 2018; Fann et al., 2018; Gardner et al., 2014a; Nordstrom and Nordstrom, 2018) compared to a hazard ratio between 1.11 and 2.51 following a single mild TBI (Barnes et al., 2018; Fann et al., 2018; Gardner et al., 2014a; Nordstrom and Nordstrom, 2018). Following repetitive mild TBI, there was a higher hazard ratio (2.81) than that seen with a single mild TBI (Nordstrom and Nordstrom, 2018), although it is important to note that this study did not adjust for other

covariates, such as LOC, gender, age, time since injury and APOE genotype. Interestingly, the risk is particularly increased for young onset dementia (dementia before 65 years old), but may still follow the dose-response relationship. For example, a 2014 study by Nordstorm *et al* showed that single mild TBI had a hazard ratio of 3.8 (95% CI: 2.8-5.2), repeated mild TBI (2 single mild TBI) had a hazard ratio of 10.4 (95% CI: 6.3-17.2) and single moderate to severe TBI had a hazard ratio of 11.4 (95% CI: 7.4-17.5) for young onset dementia (Nordstrom et al., 2014). However, when these values were adjusted for covariates (premorbid conditions affecting cognition, such as alcohol abuse), the hazard ratio for mild TBI (whether single or repeated) was 1.7 while the hazard ratio for a single moderate-severe was significantly reduced to 2.6, which, while it still validates the dose-response relationship, suggests that careful considerations should be taken regarding population based dementia studies, to avoid premorbid cognitive levels (covariates) confounding the hazard ratio. Surprisingly, when a similar study was repeated by the group in 2018, they found that the repeated mild TBI group had the highest hazard ratio of 2.8 (95% CI: 2.51-3.15) compared to severe TBI with a hazard ratio of 2.06 (95% CI: 1.95-2.19) and single mild TBI with a hazard ratio of 1.63 (95% CI: 1.57-1.70) (Nordstrom and Nordstrom, 2018), suggesting that contact-sports athletes (who are the largest contributor to numbers of repetitive mild TBI patients) may need to be wary of this risk. Despite the weaker association of a single mild TBI with dementia, it can be argued that since mild TBI is more prevalent among TBI patients, this slight increase in risk of dementia is alarming nonetheless.

Loss of consciousness (Gajavelli et al.) after TBI is regarded as a reflection of the severity of the original insult, thus it is used as an evaluation parameter in determining the GCS score post injury.The presence of LOC after TBI may also exacerbate the risk for dementia. A Multi Institutional Research of Alzheimer Genetic Epidemiology (MIRAGE) study by Guo *et al* (2000) observed a 2-fold increase risk in TBI patients with LOC compared to TBI patients without LOC (Guo et al., 2000). Similarly, a recent study by Barnes *et al* (2018) observed that the hazard ratio for mild TBI with LOC (HR=2.51 (95% CI: 2.29-2.79)) was higher than

patients with mild TBI without LOC (HR=2.36 (95% CI: 2.10-2.66)) in a cohort of military veterans (Barnes et al., 2018).

Another example of a covariate that interacts differently with dementia risk depending on injury severity is the age at which it occurs. A study by Gardner *et al* (2014) observed that mild TBI increased the risk for dementia in an older age group (65-74) compared to a younger cohort (55-64), while the inverse was true for moderate to severe TBI, with the younger cohort having a higher risk factor of 1.72 compared to the older group with a risk factor of 1.46 (Gardner et al., 2014a). Other covariates, such as time since injury and gender, may also play a role in dementia risk, however the latter covariate has not been investigated in the context of TBI severity and dementia. Investigations on the former suggest that older TBI patients (65 years and above) have an increased the risk of dementia within 10 years of injury (Gilbert et al., 2014).

3.2 Alzheimer's disease

One of the most common forms of dementia is Alzheimer's disease, accounting for approximately 75% of all dementia cases (Qiu et al., 2009). With improvements in modern medicine, the global aging population is increasing in number at an exponential rate (He et al., 2016), along with the population of AD patients (Brookmeyer et al., 2007). Despite all efforts to find a cure, researchers are still a long way from eradicating this illness. However, aging may not be the sole cause of AD, especially sporadic AD, with environmental factors such as TBI increasing this risk, especially in males (Fleminger et al., 2003c; Mortimer et al., 1991). Thus, by understanding the relationship between TBI and AD, this may provide information about the pathophysiological mechanisms driving the development of sporadic AD. In fact, one study showed the prevalence of TBI was significantly higher in the sporadic AD population than in a healthy age-matched population (Rasmusson et al., 1995) suggesting that TBI (even mild TBI) could be a great contributor to the disease progression.

Similar to dementia overall, a dose response relationship also exists for TBI and AD, with severe TBI associated with a higher risk of AD than mild TBI, while repetitive mild TBI

(regardless of the number of repeats) was associated with a higher risk compared to mild TBI (Tolppanen et al., 2017). Comparisons of risk between severe TBI and repetitive TBI have yet to be made in a population study. Interestingly, out of the five studies that have investigated a specified severity of TBI against AD development, three have looked at repetitive mild TBI (Guskiewicz et al., 2005; Leung et al., 2006; Tolppanen et al., 2017), suggesting that a history of repetitive mild TBI is most common among AD patients. Therefore, accurate predictions of AD development after single mild or severe TBI may be difficult, especially with only Tolppanen's paper comparing all three severities (Tolppanen et al., 2017).

Nevertheless, severe TBI has been predicted to reduce the age of AD onset by at least 8 years, with mild TBI also having an effect (Gedye et al., 1989). In contrast, a later study by Rasmusson (1995) suggests no significant difference in age of onset of AD between severity groups or between TBI and non-injured populations (Rasmusson et al., 1995). On the other hand, repetitive mild TBI in retired professional football players was found to decrease the age of AD onset while increasing its prevalence when compared to the age and sex matched control population, with onset of AD as early as the age of 52 in these retired athletes (Guskiewicz et al., 2005). This is a concerning notion with the high rates of participation in contact sports with the risk of repetitive mild TBI.

Unlike dementia as a whole, covariates in AD have not been well studied, with gender showing no significant influence in AD outcome (Tolppanen et al., 2017).

3.3 Chronic traumatic encephalopathy (CTE)

The other subset of dementia which is increasingly gaining attention is CTE, ever since a variant of this disease known as dementia pugilistica was first documented in boxers after Martland's observation of the 'punch drunk' phenomenon in them (Martland, 1928a). Despite the increasing number of CTE case studies among athletes (Caixeta et al., 2018; Mez et al., 2017; Omalu et al., 2010; Stein et al., 2015), studies on the risk of CTE post injury, particularly in regards to the severity of TBI, have been scarce. To our knowledge, only one population-based study has extensively studied the link between repetitive mild TBI and the development of CTE

(McKee et al., 2013b). This study by McKee and colleagues (2013) showed that 63% of a mixed population of athletes and military veterans (85 subjects in total) with a history of repetitive concussion developed CTE, with only 11% diagnosed with AD in comparison. Due to the current uncertainty of CTE diagnosis, other studies may be biased between CTE and AD, with the former only accurately diagnosed through post-mortem studies. Thus, the link between severity of TBI and CTE risk needs significantly more research.

3.4 Motor Neurone Disease (MND)

The paper by McKee et al (2013) also reported a 12% increase in motor neurone disease (MND) in their mixed population of athletes and military veterans (McKee et al., 2013b), which is slightly higher than AD, and yet, only a few studies have looked at this link between MND and TBI (Chen et al., 2007a; Raj et al., 2017). Motor neurone disease can be divided into many subtypes, with amyotrophic lateral sclerosis (Mar et al., 2013) being the most known. Thus far, studies on MND, especially ALS, have shown no known causal effect. Nevertheless, studies on TBI indicate that head injury may be a potential environmental cause for this rapidly progressive disease. Besides the 2013 Mckee study, one other earlier study in 2007 suggested a similar risk between single mild TBI and repetitive mild TBI (3 fold risk) (Chen et al., 2007a) and MND, but when time since TBI was taken into account, repetitive mild TBI within 10 years increased this risk factor to 11-fold in a New England ALS population. Conversely, Raj *et al* (2017) discovered no correlation between moderate to severe TBI and ALS in a 30 year long nationwide study involving hospitalised TBI patients in Finland (Raj et al., 2017). The scarcity of studies investigating the risk of ALS in the context of TBI severity, however, may skew the interpretation that repetitive mild TBI may be the largest contributor to the risk of motor neurone disease.

3.5 Parkinson's disease

Despite the case of the legendary boxer Muhammad Ali developing Parkinson's disease (PD) in his late forties, which was attributed to the sheer amount of repetitive concussion he endured throughout his boxing career, studies investigating PD risk in relation to TBI severity have been

contradictory. Some studies have shown no relationship between TBI and PD, with less than 1% of the study population developing PD after TBI, regardless of TBI severity (Gardner et al., 2018; Raj et al., 2017). Conversely, others suggest that TBI increases the risk of PD up to 44% within 7 years, with repetitive mild TBI having the highest HR of 1.87 compared to moderate-severe TBI with a HR of 1.50 and mild TBI with a HR of 1.24 (Gardner et al., 2015b). When further analysed, the former studies were done in a larger age range (above 18 years old), while the latter was conducted in individuals 55 years and above, suggesting that age may be a confound in PD risk prediction. Of note, a 2003 study by Bower *et al* suggest that LOC plays an important deciding factor in PD risk after TBI, where, regardless of injury severity, when combined with LOC, TBI increased the risk factor by 11 fold (Bower et al., 2003). This was supported by an earlier 1991 retrospective study, which suggested that 32% of PD patients had a history of mild TBI with LOC, despite a 37 year gap between TBI and PD onset (Factor and Weiner, 1991). Taken together, LOC after injury seem to be a crucial contributor to the risk of PD instead of TBI severity.

3.6 Non/Pre-neurodegenerative functional outcomes

Most neurodegenerative diseases in patients are diagnosed through post-mortem examination of their brains for the accumulation of disease-specific abnormal proteins (e.g. Alpha-synuclein for PD, tau for AD/CTE) (Aldag et al., 2017; Bosco et al.). While diseases like PD and ALS can be diagnosed through clinical examinations of symptomology (Postuma et al., 2015; Tao and Wu, 2017), some like CTE can only be confirmed and distinguished from AD through post-mortem examinations (Aldag et al., 2017). Nevertheless, symptoms such as cognitive decline, motor impairments and neuropsychiatric deficits may be assessed as the first form of clinical diagnosis in patients. In fact, through the assessment of cognitive decline, nearly 10% of mild cognitive impairment (MCI) patients end up being diagnosed as dementia patients later in life (Mitchell and Shiri-Feshki, 2009). Thus, the role of TBI in promoting these functional deficits chronically post-TBI is also of interest. Functional outcomes post-TBI have been extensively studied in the TBI population, particularly in terms of cognition. While these functional

outcomes may not be neurodegenerative disease per se, they may still pave the pathway towards future neurodegeneration.

Cognitive outcomes post injury are measured through many domains such as memory, learning, executive function and intellect, with memory deficits being the most common functional deficit post TBI (Barman et al., 2016; Rees et al., 2007). A few studies have looked at MCI in relation to TBI, with one study in 2005 reporting that repetitive mild TBI increases the risk of MCI diagnosis by 5 fold, while general memory impairment was associated with a three-fold increased risk after repeated concussion (Guskiewicz et al., 2005). Another study in 2016 showed that the age of onset of cognitive impairment was greatly reduced with increasing severity of TBI, especially when combined with MCI diagnosis, where the cognitive decline in non-TBI MCI patients had a later age of onset compared to MCI patients with a history of mild TBI, by at least 4 years (Li et al., 2016). Taken together, a history of TBI, even of mild severity, is able to impair cognition. As previously mentioned, memory is greatly impaired after TBI, however, memory only seems to be impaired post repetitive mild TBI and severe TBI coupled with LOC (Esopenko et al., 2017; Gardner et al., 2017; Himanen et al., 2006; Kaup et al., 2017), with no significant impairment seen post mild TBI with or without LOC (Albrecht et al., 2016; Himanen et al., 2006; Kaup et al., 2017; Rapoport et al., 2008), suggesting that the severity of TBI affects memory outcome. Learning deficits, which have been less investigated in this context, displayed similar conditions as memory impairment, where the link was only significant in repetitive mild TBI and severe TBI (Himanen et al., 2006; McMillan et al., 2017).

Executive function includes a range of cognitive parameters such as reaction speed, impulsivity, inhibition, motivation and flexibility. To date, a number of studies have looked at the link between a history of TBI and impairment in executive function (Alosco et al., 2017; Kaup et al., 2017; List et al., 2015; Palacios et al., 2013; Pedersen et al., 2014a; Rapoport et al., 2008; Wilde et al., 2016). When executive function and intellect were studied in a population of retired hockey players, those who sustained a higher number of concussions showed a greater decline in response speed, visual processing and recall memory (Pedersen et al., 2014a).

Similarly, another study in military veterans showed that a lifetime history (5 years or more) of multiple mild TBI or moderate-severe TBI was linked to executive function deficits and reduced cognitive speed, while those with a single mild TBI performed equally to non-injured controls (Kaup et al., 2017). Interestingly, impulsiveness seems to decrease with a history of repetitive mild TBI, as seen in a professional fighter population (Banks et al., 2014). To conclude, while cognition is significantly impaired after TBI, this is only true in repetitive mild and severe forms of TBI, therefore supporting the higher risk of neurodegenerative disease seen in these more severe groups.

Other functional outcomes, such as motor impairment and neuropsychiatric outcome (anxiety or depression), are less studied in conjunction with TBI severity. Higher rates of mood impairment, especially depression, is seen in national football league players who had sustained repetitive mild TBI (Alosco et al., 2017; Hart et al., 2013) compared to non-concussed players. However, there is a lack of studies on this outcome. Similarly, retired boxers with sustained repeated concussion display motor coordination impairments (Bang et al., 2016). While motor-related neurodegenerative diseases, like PD and ALS, have been shown to be correlated with repetitive mild TBI only (De Beaumont et al., 2012), due to the lack of studies investigating other TBI severities, it is difficult to truly interpret these results.

4. Pre-clinical studies

The best approach towards any intervention for a clinical problem is through pre-clinical investigations (*in vitro* and *in vivo*). Understanding the pathology, the outcomes and the safety and efficacy of treatments that can interact and intervene the pathology have been carried out by researchers for decades by utilising experimental models, particularly murine models (rodents), with no functional outcomes yet reported in large animal models. Thus far, impairments in memory, learning and executive function, as well as deficits in motor activity and neuropsychiatric function, have been thoroughly investigated in pre-clinical studies of TBI, though the results of these investigations have been somewhat contradictory. The different

injury models (focal versus diffuse), age at TBI induction (young versus old) and animal species (Sprague Dawley versus B6 mice) have made translation across labs difficult. Furthermore, studies comparing the impairments post different severities of TBI are still scarce. Thus, could one severity of TBI result in a greater cognitive impairment than the other or could they work in opposite directions? Is there a severity threshold that defines the type or extent of the post injury impairment and long-term outcome? Satisfying these questions will be the best approach towards TBI intervention.

4.1 Cognitive outcomes

Following a parallel pattern to clinical studies, pre-clinical investigations on the functional outcomes post-different severities of TBI have largely focused on cognition and often been related to future dementia outcomes.

Learning and memory, particularly spatial and working memory, are typically assessed through maze tasks such as the Morris water maze (MWM) and Barnes maze (Olivera et al., 2015), which have a number of training days for learning and a probe day for memory (Harrison et al., 2009). Occasionally, these more complex maze tasks will be coupled with an easier, but cruder, measurement of spatial memory known as the Y-maze test. Using the Y-maze, decreases in spatial memory either through spontaneous alteration behaviour or novel preference have been observed post controlled cortical impact (CCI) (Tucker et al., 2016) and following weight drop injury (Heim et al., 2017), but not all studies have been consistent (e.g.(Rachmany et al., 2013)), suggesting that cognitive outcomes post diffuse TBI may be subtle and require behavioural assessment with complex cognitive measurements.

Previous literature suggests that there is an effect of TBI severity on cognitive outcomes, with repetitive mild TBI being more associated with worsened learning and memory performance than a single mild TBI (Gao et al., 2017; Mouzon et al., 2014). Following repetitive mild TBI, animals show slower learning during the training phase of the MWM/BM, as well as memory deficits in identifying the location of the hidden platform/hole in the maze on probe day, when compared against shams, regardless of the TBI model (Briggs et al., 2016;

Gao et al., 2017; Lynch et al., 2016; McAteer et al., 2016; Zhang et al., 2015) These deficits are chronic, persisting up to 6 months in the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) model (Chen et al., 2017a; Nolan et al., 2018b), up to 6 months after CCI (Lynch et al., 2016; Petraglia et al., 2014; Zhang et al., 2015), up to 3 months post fluid percussion injury (FPI) (Shultz et al., 2012; Tan et al., 2016) and up to 25 days after weight-drop (Briggs et al., 2016). In the longest timepoint investigated to date, impairments were still detectable at 18 months post CCI injury (late adulthood in rodents) (Mouzon et al., 2014), suggesting cognitive deficits may persist or re-emerge at later timepoints (18 months), which may have implications for dementia risk following repetitive mild TBI. However, not all studies have been consistent, with several showing no changes in cognitive outcome measured on the MWM acutely (7 days) or chronically (4- 6 months) post CCI (Laurer et al., 2001; Uryu et al., 2002; Yoshiyama et al., 2005). Upon closer inspection, these studies were conducted in mice of 9 to 12 month old of age. Since age of injury onset can influence the TBI outcome in humans, as discussed previously, it is reasonable to hypothesise that a similar effect may occur in rodents and that induction of repetitive mild TBI in later adulthood may effect cognitive function differently than one that occurs earlier in life. However, caution should be given when comparing repetitive mild TBI between clinical and preclinical models, mainly due to its heterogeneity in terms impact severity, frequency and recovery time between impacts that differs not only between the clinical and preclinical models but within them too.

Nevertheless, when compared to repetitive mild TBI, the cognitive deficits observed following a single mild TBI have been less pronounced (Gao et al., 2017; Mouzon et al., 2014; Webster et al., 2015), but nonetheless observable, affecting both learning (Mouzon et al., 2014) and memory (Rachmany et al., 2013; Saber et al., 2017). These impairments have been weakly demonstrated at earlier stages (7 days to 1 month) (Rachmany et al., 2013) but more strongly displayed chronically (6-18 months) (Mouzon et al., 2014) post single mild injury when compared to sham counterparts, suggesting persistent cognitive deficits may develop even after single mild TBI. As for moderate to severe TBI, acute studies (less than 1 month) have shown

significant deficits in recognition memory on the novel object task as early as 24 hours post weight drop injury (Chen et al., 2016) as well as displayed impairments in retention memory on the barnes maze within a week post FPI (Krishna et al., 2017) and on the MWM up to 1 month post CCI injury (Geddes et al., 2017). Additionally, Ouyang *et al* (2017) reported persistent learning and memory impairments acutely on the MWM were observable from day 1 post FPI up to 13 days post injury (Ouyang et al., 2017). However, learning and memory measurements in acute studies may be influenced by early post surgery motor deficits (Ouyang et al., 2017) and therefore, chronic studies may be more informational regarding these cognitive impairments. Unfortunately, chronic studies beyond 1 month post-injury have been scarce and inconsistent, with one study showing no changes in cognitive outcome on the MWM up to 6 weeks post moderate to severe CCI injury (Tajiri et al., 2013) and, conversely, two others demonstrating the presence of spatial and working memory deficits as assessed through MWM up to 3 months after either CCI or FPI injury (Byrnes et al., 2012; Kokiko-Cochran et al., 2016). Although moderate to severe TBI may be speculated to be more damaging than mild TBI, based on the handful of studies conducted, it appears that repetitive mild TBI may be more detrimental for long-term cognitive outcomes. However, due to the scarcity of long-term studies on cognitive outcomes following moderate to severe TBI, particularly in different models of injury, it is not possible to come to a clear conclusion and future research is needed.

Additionally, other aspects of cognitive function, such as executive function, is also known to be affected following TBI (Ozga et al., 2018). The term 'executive function' includes behaviours such as attention, impulsivity, flexibility and decision-making, that may be measured individually after TBI through rule shift assay (Chou et al., 2016b), attentional set shift task (AST) (Bondi et al., 2014), barnes maze (Taib et al., 2017) or pairwise visual-discrimination task (Robinson et al., 2018), or otherwise collectively post injury in the 5 choice serial reaction time task (5CSRT) or go/no-go task (Hehar et al., 2015; Ozga et al., 2018). Mild TBI has displayed impairments in attention, inhibition, impulsivity and cognitive flexibility mainly acutely (13 days) (Hehar et al., 2015) and chronically (up to 3 months) (Taib et al., 2017;

Vonder Haar et al., 2016) post CCI injury, with one study showing executive impairments acutely (2 days) post weight drop injury on the go/no-go task (Mychasiuk et al., 2015), while moderate to severe TBI exhibited deficits in more facets of executive function such as impairments in discrimination, cognitive flexibility, attention, impulsivity and motivation as early as 13 days up to 3 months post CCI injury (Crane et al., 2012; Robinson et al., 2018; Vonder Haar et al., 2016). The injury severity study by Vonder Haar *et al* (2016) found that moderate to severe TBI animals displayed deficits in attention, impulsivity and motivation on the 5CSRT up to 14 weeks post CCI injury but only saw deficits in impulsivity in the mild CCI group (Vonder Haar et al., 2016), suggesting executive function impairments post injury has a dose-response relationship. This relationship is further supported by an earlier study showing severity-dependent attention and flexibility impairments on the AST a month post CCI injury (Bondi et al., 2014). Unlike memory and learning impairments, executive function post experimental repetitive mild TBI may not have been investigated thus far and requires further research, given the executive deficits seen in repetitively concussed athletes (Alosco et al., 2017; Esopenko et al., 2017).

4.2 Motor outcomes

A dose-response relationship may exist against motor outcomes chronically post injury in a rodent model. This was supported by Erturk and his colleagues (2016) in their 8-week investigation of TBI severity effects on the balance beam and open field after CCI injury in mice, in which they showed only the moderate TBI animals displayed persistent but recovering motor deficits with no changes seen in mild TBI at any timepoint post injury (Erturk et al., 2016). While, Thomsen and his group also demonstrated a dose response relationship exist after repetitive mild CCI injury whereby the greater the number of hits, the more severe and persistent the motor impairment displayed; 5 weekly consecutive mild TBIs led to severe motor deficits on the rotarod that persisted up to 24 weeks (Thomsen et al., 2017). Similarly, an earlier study concluded that a second mild TBI within 24 hours exacerbated the first mild injury, leading motor impairments to manifest in mice after repetitive mild CCI injury (Laurer et al.,

2001) but Uryu and colleagues (2002) found no change in motor performance at 16 weeks following single mild or repetitive mild TBI in a similar CCI model (Uryu et al., 2002), further supporting a dose-response relationship in motor outcomes.

Additionally, Byrnes *et al* (2012) showed that vehicle treated mice with moderate TBI had persistent motor impairments up to 3 months post-CCI injury (Byrnes et al., 2012) which was supported by another study in 2016 that observed similar persistent motor deficits on the rotarod after moderate FPI injury (Kokiko-Cochran et al., 2016). A more recent study (2017) by the Shultz laboratory on Long Evans rats concluded motor impairments up to 12 weeks after moderate FPI-induced TBI, which was attributed to an increase in phosphorylated TDP-43 protein, a hallmark pathology of MND (Wright et al., 2017a).

Thus, underlying pathophysiological mechanisms relevant to neurodegenerative disease may drive the manifestation of motor impairments at these chronic timepoints. It remains to be investigated, however, whether these pathophysiological mechanisms differ as a function of the original injury severity. Additionally, characterising the temporal profile of these changes will be important, in order to assess whether these mechanisms worsen from time of injury or are a later result of the disease process.

To note, the contrasting motor outcomes between the repetitive mild CCI injury in Laurer's study with Uryu's study, despite a similar injury procedure to induce repetitive mild TBI (2 consecutive hits with 24 hours apart), could be attributed either to the strain of the animal (transgenic wild type littermates versus C57BL/6 standard mice) or the age of TBI induction (9 months versus 10-week-old). The strain or age of the animal can greatly affect the results of the study due to the differences in the neuroprotective nature/ vulnerability of the brain (Reid et al., 2010; Rowe et al., 2016). For example, at least in rats, Reid *et al* (2010) clearly showed a strain difference in injury susceptibility against functional and neuropathological outcomes between the Fisher 344 and SD rats post FPI injury, with the former displaying greater deficits and neuropathology overall (Reid et al., 2010). Likewise, Rowe *et al* (2016) suggest that age of injury may influence the outcome post injury, whereby SD rats injured (moderate FPI) at early

developmental age (postnatal day 17, 35 or 2 months of age) displayed motor and cognitive impairments which was not present in rats injured at adulthood (4 and 6 months) but the latter group displayed anxiety-like phenotype instead (Rowe et al., 2016), suggesting differences in vulnerability towards injury due to age in rats, a concept which may be extended to mice as well. Therefore, the results by Laurer and colleagues may be more informative, as they used a more standard strain and age of mice.

While further investigations are necessary, particularly at long-term follow up timepoints, taken together, these studies do seem to indicate a dose response relationship, with moderate TBI having the worse motor impairment outcome and single mild TBI displaying the least, regardless of strain and age of injury onset.

4.3 Neuropsychiatric outcomes

Anxiety and depression are the two most common types of neuropsychiatric outcomes displayed by TBI patients and neurodegenerative disease patients alike (Lyketsos et al., 2007). Depressive-like behaviour, even up to 3 months post-injury, has been consistently observed in preclinical studies of repetitive mild TBI, regardless of rodent species or type of TBI (Bajwa et al., 2016; Briggs et al., 2016; Petraglia et al., 2014; Shultz et al., 2012; Tan et al., 2016), similar to reports from contact-sport athletes (Hart et al., 2013; Vargas et al., 2015a). Even single mild TBI has been shown to be associated with depressive-like behaviour up to 3 months post-injury (Bajwa et al., 2016; Milman et al., 2005). Mice demonstrated elevated levels of depressive-like behaviour on the tail suspension test at 90 days following a single mild closed head injury (Bajwa et al., 2016). Interestingly, repetitive mild TBI was not associated with more severe depressive-like behaviour at this timepoint. In contrast, moderate TBI has not been shown to be associated with increased depressive-like behaviour at long-term timepoints. While studies show increased immobility on both the tail suspension test and forced swim test following moderate to severe TBI at less than 7 days post injury, this effect was not seen at later timepoints (Fenn et al., 2014; Kuo et al., 2013). Similarly, Bajwa *et al* (2016) showed that there was no increase in immobility in animals who had experienced a moderate TBI inflicted by CCI

compared to sham animals on the tail suspension test at 90 days. Thus, while both mild and mild repetitive TBI may be associated with long-term depression following TBI, more severe TBI may play a role in more acute, but not chronic, timepoints.

On the other hand, anxiety-like behaviour has been quite contradictory between studies (Nolan et al., 2018b; Petraglia et al., 2014; Shultz et al., 2012). In multiples studies, animals have shown significant anxiety (measured as shorter time spent in open arms of elevated plus maze) up to 8 weeks following repetitive mild TBI (Broussard et al., 2018; Shultz et al., 2012) with McAteer *et al* (2016) showing a trend towards decrease time spent in inner portion of the open field test (OFT) at 12 weeks post repetitive mild TBI (McAteer et al., 2016), but studies by Petraglia (2014) and Nolan (2018) showed a shift towards increased risk taking behaviour (more time spent in open arms of elevated plus maze (EPM) at 1 month post injury (Nolan et al., 2018b; Petraglia et al., 2014). In the Petraglia *et al* study, this behavioural phenotype persisted for up to 6 months post injury. When compared against Petraglia's study, the differences in rodent species; mice versus rats in Shultz's study, the amount of repetitive hits; 6 impacts daily for a week versus 3 or 5 hits with 5 day intervals in Shultz's study and the TBI model; CCI versus FPI used in the study by Shultz *et al* (2012) may influence the persistent anxiety (decreased time and entries in the open arms of EPM) seen in their study (Shultz et al., 2012), suggesting that a greater cortical damage (represented by amount of hits) is needed for the shift in anxiety behaviour. In support, at 1 month post injury, Nolan's study utilising mice with daily hits for 5 days on the CHIMERA model also created this shift in anxiety (Nolan et al., 2018b) while mice with a single mild TBI induced by the weight drop model showed increased anxiety (Rachmany et al., 2013), suggesting that even diffuse injury may cause this shift towards risk-taking behaviour in mice when the severity of the TBI is increased. However, when the severity was kept constant but the type of TBI model used differed, again there is a shift in anxiety; at 1 month post injury, animals who had undergone mild TBI induced by FPI exhibited risk taking behaviour, with TBI animals spending more time in the open arms of the EPM post injury compared to shams (Saber et al., 2017), thus suggesting a significant influence

of TBI model on anxiety outcome post-mild TBI but a significant influence of TBI severity within a single TBI model used.

Thus far, studies on neuropsychiatric outcome post moderate to severe TBI have been scarcely investigated in preclinical models and this remains an urgent area for future research. One study showed that moderate to severe diffuse TBI (weight drop) in SD rats have persistent (up to 42 days) depressive-like phenotype post injury (Fromm et al., 2004), while another study indicated this persistence in depression and even anxiety to last up to 3 months post severe FPI (Jones et al., 2008). However, a study in mice suggest that moderate to severe TBI after weight drop may display no changes in depression or anxiety acutely post injury (Schwarzbold et al., 2010), suggesting a probable species-dependent susceptibility to diffuse TBI, therefore, when moderate to severe CCI injury is subjected to mice, deficits in forced swim test (depression) and EPM become evident at 21 days post injury (Washington et al., 2012).

Taken together, neuropsychiatric outcomes post injury may be more sensitive to the variation in experimental TBI conditions across studies compared to cognition or motor outcomes. This reflects the neuropsychiatric contradictions in the human population after TBI that is dependent more than just the TBI severity or type of injury, but is influenced by a range of other environmental factors such as substance abuse, alcoholism, premorbid behaviour/disorder and social support (Ahmed et al., 2017). This suggest that a dose response relationship should be cautiously implied for depression and anxiety measures post injury in both clinical and preclinical models.

5. Conclusion and Future studies

Based on review of the current literature, a significant gap in TBI knowledge still exists, particularly in preclinical research. The scarcity of research regarding long-term functional outcomes in experimental models of moderate/severe TBI may underrepresent/downplay its cruciality in clinical studies, therefore preventing intervention studies to successfully proceed. Additionally, while there are multiple preclinical studies of long-term outcomes following both

repetitive mild TBI and single mild TBI, there is still a lack of studies comparing the TBI severity effect within a single cohort. This is particularly important, given that this review has shown that multiple factors, such as rodent species, TBI induction model, age of TBI onset and amount of repetitive hits, may all influence the long-term functional changes seen. Finally, only a small number of studies have actually looked at truly chronic timepoints (beyond the 1 month timepoint) of functional deficits in any model of TBI, which may obscure the effects of age seen in humans, and fewer studies still have investigated underlying pathophysiological mechanisms such as tauopathy in dementia, alpha-synuclein in PD and TDP-43 abnormalities in MND at these timepoints. Therefore, future studies should investigate both functional deficits and neuropathological change at chronic timepoints (beyond 6 months at least) in preclinical models of TBI. Only then can we begin our journey towards effectively treating TBI patients to improve long term functional outcomes post injury and potentially even lower the risk of neurodegenerative disease following injury.

Chapter 2

Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury

Statement of Authorship

Title of Paper	Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury
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Principal Author

Name of Principal Author (Candidate)	Alina Arulsamy		
Contribution to the Paper	Performed analysis of behavioural data and brain samples, interpreted all data and wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/02/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury.

Running title: Characterisation of the chronic effects of experimental diffuse traumatic brain injury

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AA, JT and HC were involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

Abstract

TBI is a significant risk factor for the development of dementia, with the interaction between structural damage from TBI and neuroinflammation potentially driving this relationship. This study investigated the early chronic post-TBI neuroinflammatory response and its relationship to both neurodegenerative pathology and functional impairment up to 3 months post-injury. Sprague-Dawley rats underwent either sham surgery or the Marmarou model of diffuse moderate-severe TBI. At 1-month and 3-months post-injury, a functional battery encompassing motor function, depressive-like behaviour, anxiety and cognition was performed. Western blot and immunohistochemical analysis assessed a range of inflammatory, neurodegenerative and oxidative stress markers. At both 1 and 3-months post injury, depressive-like behaviour was significantly increased in TBI animals, with TBI animals also exhibiting impaired cognitive flexibility at 3 months, although learning and memory remained intact. This was accompanied by a significant decrease in markers of synaptic integrity and astrocytic and microglia number within the pre-frontal cortex at 1-month post-injury, although this resolved by 3-months post-injury. In contrast, minimal pathology was evident within the hippocampus at 1 month, with only a decrease in neurofilament-light seen at 3 months post-injury. Thus, following a moderate-severe diffuse injury, the pre-frontal cortex is most vulnerable to early neuro-structural changes, which may re-emerge or progress to other areas such as the hippocampus at more chronic stages, which could predispose individuals to early dementia.

Key words: neurodegeneration, cognition, depression, head injury, dementia

1. Introduction:

Traumatic brain injury (TBI) represents one of the leading causes of mortality and disability worldwide. The Centre of Disease Control and Prevention stated that, in 2010 alone, there were approximately 2.5 million emergency department visits, hospitalisations and deaths due to TBI (Faul et al., 2010; Frieden et al., 2014). There is increasing evidence to suggest that neuronal injury is ongoing following a TBI (Carron et al., 2016; Gao and Chen, 2011; Sato et al., 2001), and that moderate-severe TBI may lead to progressive neurodegeneration, such as dementia and associated cognitive and behavioural deficits. Population based studies following patients with moderate-severe TBI showed these functional deficits persisting years later, even after motor function recovery (Dikmen et al., 2003; Gupta and Taly, 2012; Stocchetti and Zanier, 2016). An Australian health survey of TBI cases reported an overall decrease in mental health quality and elevated depression levels when compared to a matched non-TBI cohort, even up to 15 years after injury (Hawthorne et al., 2009).

Indeed, following a focal injury, lesion volume was found to increase nearly 5 fold over one-year post-injury (Loane et al., 2014), whereas, following a mixed focal/diffuse injury induced by lateral fluid percussion, cortical and hippocampal tissue loss increased significantly from one week to one year post-injury (Smith et al., 1997). This is supported by clinical imaging studies, which have shown progressive white matter damage, particularly within the frontal and temporal regions, as well as loss of cortical grey matter, up to a year post-injury (Bendlin et al., 2008), in line with reports of progressive reductions in brain volume as assessed up to 14 months post-TBI (Trivedi et al., 2007).

The exact mechanisms that drive this ongoing neuronal injury are yet to be fully elucidated, with the development of an aberrant persistent chronic neuroinflammatory response thought to be one key mechanism (Corrigan et al., 2016a). Indeed, multiple studies have demonstrated that a neuroinflammatory response may persist following resolution of the acute effects of a TBI, with inflammatory markers present in the brain parenchyma, serum and cerebrospinal fluid of TBI patients at chronic time points (months to years later) (Juengst et al., 2014; Kumar et al.,

2015b; Ramlackhansingh et al., 2011; Smith et al., 2013). In rodents, microglial activation has been demonstrated up to one-year following a focal TBI, with associated progressive lesion expansion, hippocampal degeneration, myelin loss and oxidative stress (Loane et al., 2014). Although a number of studies have shown progressive neuronal loss up to one year post-injury, a more detailed examination of the events that occur in the sub-acute and early chronic stages post-TBI that may promote this ongoing neuronal injury have received less attention. Furthermore, these studies have been predominantly conducted utilising focal (Loane et al., 2014) or mixed focal models (Smith et al., 1997), rather than a purely diffuse injury. As such, this study sought to investigate the effects of a moderate-severe diffuse TBI at 1 and 3 months post-injury on synaptic and axonal integrity and neuroinflammation, as well as on functional outcome.

2. Results:

2.1 *Motor outcome*

Motor outcome was assessed weekly up to 3 months (Fig. 1A) on the rotarod. Sham and TBI animals showed no significant differences in their pre-training rotarod scores but a significant injury effect on the scores was seen in the weeks following the injury ($F_{1,19} = 5.146$, $p=0.035$). TBI animals showed a significantly impaired rotarod scores when compared to shams (67.8±13.47 secs vs 114.3±4.23 secs in sham animals, $p<0.0001$) at 24 hours post injury (indicated by week 1 on Fig 1A). However, by the third week (day 15) post-injury, TBI animals had returned to sham levels, (112.7±4.46 secs vs 117±2.09 secs, $p>0.9999$) and maintained this for the rest of the testing period.

2.2 *Locomotor activity*

General locomotor activity was assessed as the distance travelled in the open field test (OFT). At 1 month post-injury (Fig. 1B(i)), TBI animals showed no difference in locomotor activity compared to shams (39.36±1.6m vs 42.35±2.3m in shams; $t(32) = 1.089$, $p=0.2843$), but at 3 months post-injury (Fig. 1C(i)), there was a significant decrease in locomotor activity in the TBI animals when compared to shams (23.6±4.0m vs 35.5±2.4m; $t(23) = 2.479$, $p=0.0209$).

2.3 *Anxiety-like behaviour*

Anxiety-like behaviour was measured as time spent in centre of OFT and time spent in the open arms of the elevated plus maze (EPM). No significant differences in time spent in centre of OFT and open arms of EPM were seen between the TBI animals and sham animals at 1 month (17.9±2.9 secs vs 15.9±2.8 secs in shams; $t(32) = 0.485$, p=0.6314) and (95.0±10.8 secs vs 86.7±7.9 secs in shams; $t(32) = 0.591$, p=0.5587) respectively (Fig. 1B (ii) and (iii)). Similarly, at 3 months post-injury, no significant differences were seen between groups in time spent in centre of OFT (5.9±2.1 secs vs 7.5±3.3 secs in shams; $t(23) = 0.427$, p=0.6735) (Fig. 1 C(ii)) as well as time spent in open arms of EPM (64.8±14.7 secs vs 80.0±11.7 secs in shams; $t(23) = 0.798$, p=0.4329) (Fig. 1C(iii)).

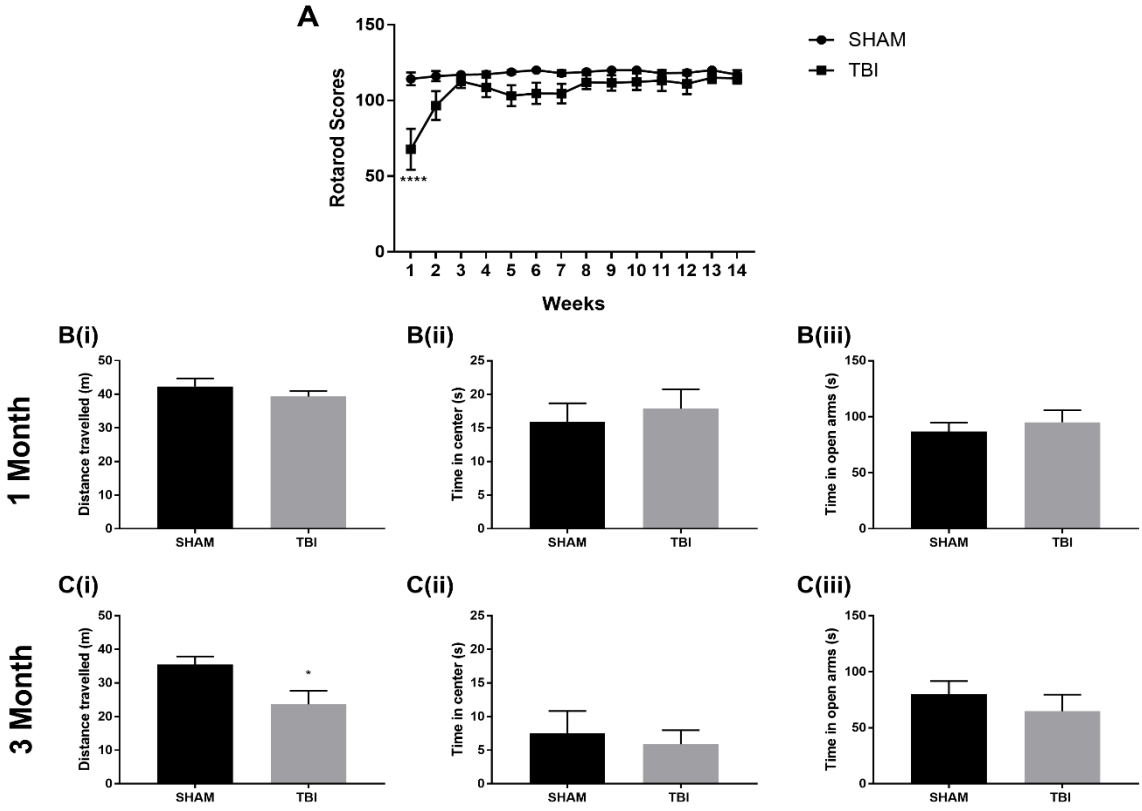

Figure 1: Functional outcomes measured post injury. A) Motor outcome as measured on the rotarod, weekly for 3 months. Locomotor activity as measured on the open field at B(i)) 1 month and C(i)) 3 months. Anxiety-like behaviour as measured in the open field at B(ii)) 1 month and C(ii)) 3 months and on the elevated plus maze at B(iii)) 1 month and C(iii)) 3 months. Graphs represent the mean ± SEM, (n= 13-19 per group; ****p<0.0001,*p<0.05 compared to shams).

2.4 *Depressive- like behaviour*

Depressive-like phenotype was assessed based on the immobility time in the forced swim test (FST). TBI animals spent more time immobile than shams at 1 month post-injury (125.8±7 secs vs 99.2±9.4 sec in shams; $t(32)= 2.32$, $p= 0.0269$) (Fig. 2A), with this persisting at 3 months post-injury (185.6±6.8 secs vs 151.2±8.4 secs in shams; $t(23)= 3.217$, $p=0.0038$) (Fig. 2B).

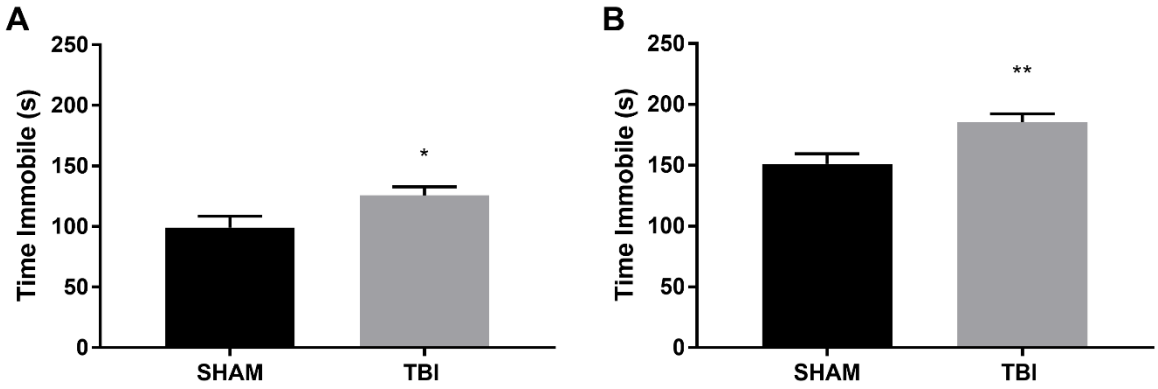

Figure 2: Depressive-like behaviour as measured in forced swim test at A) 1 month and B) 3 months. Graphs represent the mean ± SEM, (n= 13-19 per group; **p<0.01, *p<0.05 compared to shams).

2.5 *Cognition*

Cognitive outcome was assessed using the Y-Maze for spatial memory and Barnes maze for learning, memory and cognitive flexibility (ability to reprogram previously learned task) (Fig. 3). Y-Maze was performed at 1 month and 3 months post-injury, while the Barnes maze was only performed on the 3 month animals. Spatial working memory in the Y-Maze showed no significant changes in novel preference between the TBI group and the sham control group at any of the time points post-injury; 1 month (0.37±0.03 vs 0.42±0.03 in shams, $t(32) = 1.009$, $p=0.3205$), 3 month (0.37±0.04 vs 0.39±0.03 in shams, $t(23) = 0.326$, $p=0.7475$) (Fig. 3A-B). On the Barnes Maze, no significant differences were noted in time taken to locate the escape box on any of the training days during the acquisition phase ($F_{1,23} = 0.049$, $p=0.8276$)(Fig. 3C). Nor was there any difference in ability to locate the old escape box on the probe day (shams

27.8±12.6 vs TBI 14.0±4.2 secs; *t*(23) = 1.076, p= 0.293) (Fig. 3D). In terms of learning the location of the new escape box on probe day, there was a trend of injury effect ($F_{1,23}$ = 3.979, p=0.0581). The sham animals showed greater cognitive flexibility taking a significantly shorter time on Trial 1 compared to TBI animals (63.0±16.3 secs vs 24.0±4.6 secs in shams, p=0.014), although both groups had similar times on trial 2 (24.0±7.7 secs vs 15.24±3.1 secs in shams, p=0.528) (Fig. 3E).

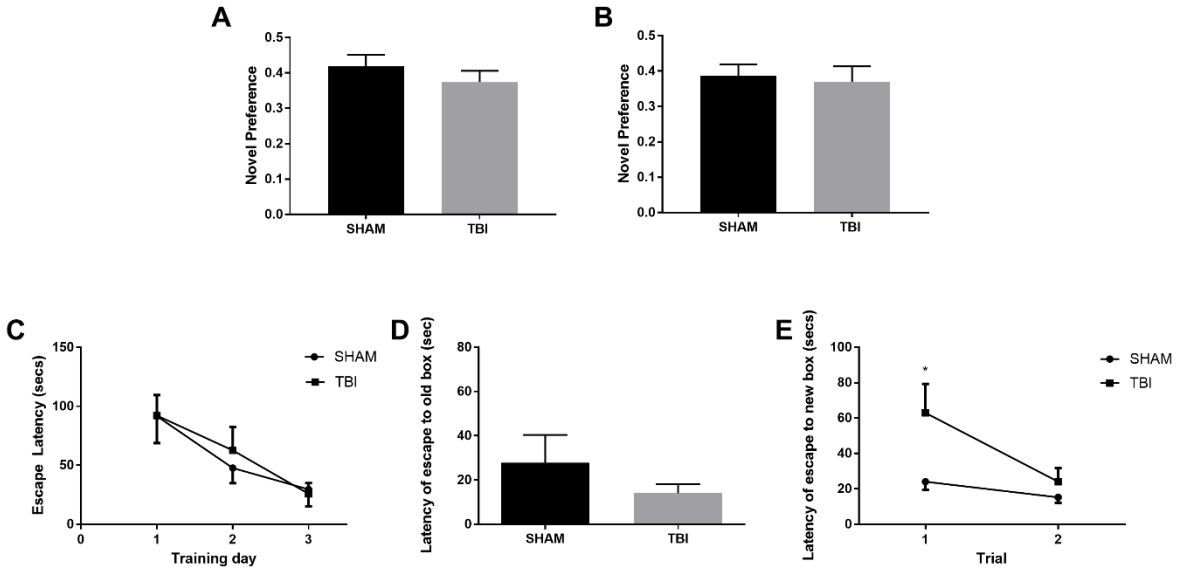

Figure 3: Cognition assessed through Y-maze for spatial working memory at A) 1 month and B) 3 months post-injury and the Barnes maze at 3 month post-injury (C-E). For the Barnes Maze, C) learning ability in the acquisition phase, D) recollection memory during the probe trial and E) cognitive flexibility on probe day are shown. All graphs show mean ± SEM, (n= 13-19 per group; *p<0.05 compared to shams).

2.6 *Acute neuroinflammatory changes in prefrontal cortex (PFC) post-TBI*

Levels of inflammation were assessed by counting the number of cells that were immunopositive for GFAP (glial fibrillary acidic protein) (Fig. 4), a structural protein in astrocytes and IBA1 (ionized calcium binding adaptor molecule 1) (Fig. 5), a calcium binding protein seen in microglia within the PFC and hippocampus. At 1 month post injury, GFAP immunopositive staining (GFAP+ve) was decreased within the PFC in TBI animals

(135.8±14.39 cells/mm^2) compared to shams (193.4±13.48 cells/mm^2) *(t*(7)=2.92, p=0.019) (Fig. 4C). However, the number of GFAP+ve cells in the hippocampus of TBI animals (193.0±22.9 cells/mm^2) and shams (207.5±5.91 cells/mm^2) did not differ significantly between groups (*t*(7)=0.55, p=0.601) (Fig. 4D). At 3 months post injury, the number of GFAP+ve cells did not significantly differ between the shams and TBI in either the PFC (62.19±5.67 cells/mm^2 vs 61.61±11.58 cells/mm^2 in shams, *t*(6)=0.052, p=0.96) or the hippocampus (453.5±29.01 cells/mm^2 vs 461.6±22.11 cells/mm^2 in shams, *t*(7)=0.214, p=0.84) (Fig. 4G-H).

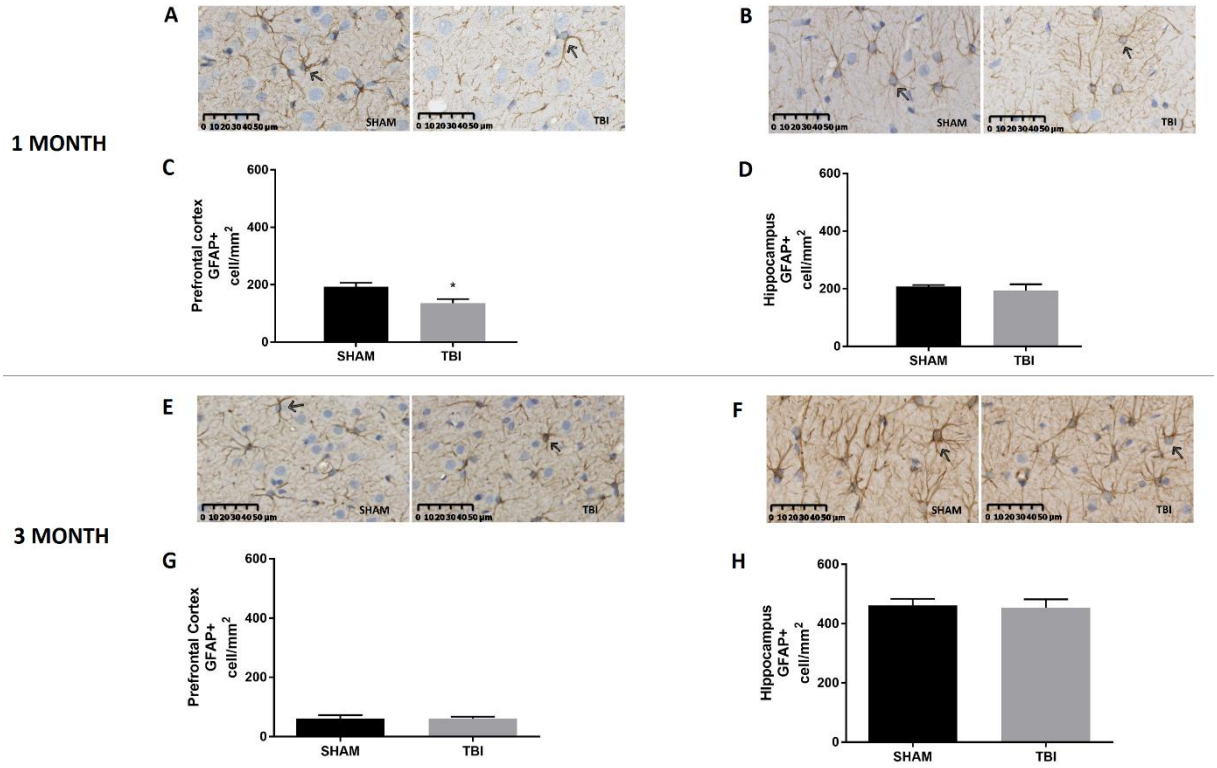

Figure 4: Representative images of GFAP staining within the A,E) PFC and B,F) hippocampus at A-B) 1 month and E-F) 3 months post-injury, as well as their respective cell counts at C-D) 1 month and G-H) 3 months. Graphs represent the mean ± SEM, (n= 4-5 per group; *p<0.05 compared to shams).

Similarly, the number of IBA1+ve cells in the PFC of TBI animals (73.84±5.48 cells/mm^2) was significantly decreased compared to shams (94.45 ±6.70 cells/mm^2) *(t*(7)=2.33, p=0.049) at 1 month post-injury (Fig 5C). In contrast, the hippocampus showed no significant differences in IBA1+ve staining in the TBI animals (66.78±5.03 cells/mm^2) compared to shams (86.99±12.2

cells/mm^2) (*t*(7)=1.54, p=0.176). By 3 months post-injury, there was no significance difference in IBA1+ve staining in the PFC (38.54±4.72 cells/mm^2 vs 32.39±3.96 cells/mm^2 in shams, *t*(6)=0.89, p=0.409) or the hippocampus (254.9±17.28 cells/mm^2 vs 235.9±18.5 cells/mm^2 in shams, *t*(7)=0.75, p=0.478) between the groups (Fig. 5G-H).

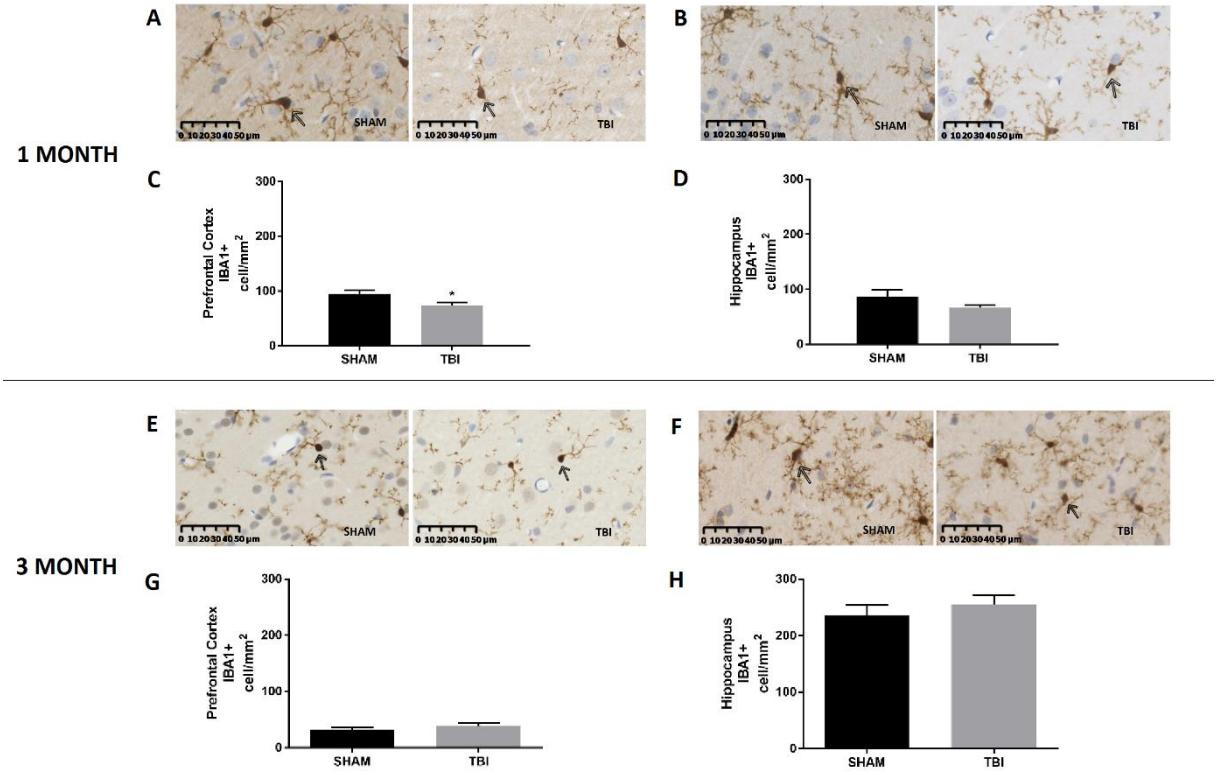

Figure 5: Representative images of IBA1 staining in the A,E) PFC and B,F) hippocampus at A-B) 1 month and E-F) 3 months, as well as their respective cell counts at C-D) 1 month and G-H) 3 months. Graphs represent the mean ± SEM, (n= 4-5 per group; *p<0.05 compared to shams).

2.7 *Evaluation of neuronal and synaptic integrity*

Neuronal and synaptic structural damage post injury was assessed using a variety of markers; PSD-95 (postsynaptic density protein 95) and synaptophysin for assessing synaptic integrity, NF-L (neurofilament light chain) and NF-H (neurofilament heavy chain) for assessing neurofilament structure and axonal stability and MBP (myelin basic protein) for assessing neuronal myelination stability. In the PFC, at 1 month post injury, the relative density of PSD-95 and synaptophysin were significantly reduced in the TBI animals compared to shams

(0.964±0.214 vs 1.721±0.041, $t(6)= 2.64$, p=0.039 and 2.103±0.469 vs 3.623±0.229, $t(5)= 2.59$, p=0.049, respectively) (Fig. 6). This had resolved by 3 months post-injury, with similar values reported in TBI and sham animals; PSD-95 (1.717±0.322 vs 1.738±0.031 in shams, $t(6)=0.049$, p=0.962), synaptophysin (1.77±0.268 vs 1.72±0.192 in shams, $t(8)=0.144$, p=0.889). In comparison, in the hippocampus, there were no significant differences in the relative density of PSD-95 and synaptophysin at either 1 month; PSD-95 (0.474±0.056 vs 0.369±0.037 in shams, $t(8)=1.57$, p=0.154), synaptophysin (0.786±0.085 vs 0.973±0.107 in shams, $t(6)=1.363$, p=0.222) or 3 months; PSD-95 (0.239±0.028 vs 0.272±0.024 in shams, $t(7)=0.877$, p=0.41), synaptophysin (0.519±0.085 vs 0.515±0.123 in shams, $t(8)=0.022$, p=0.983) post-injury (Fig. 6E-H).

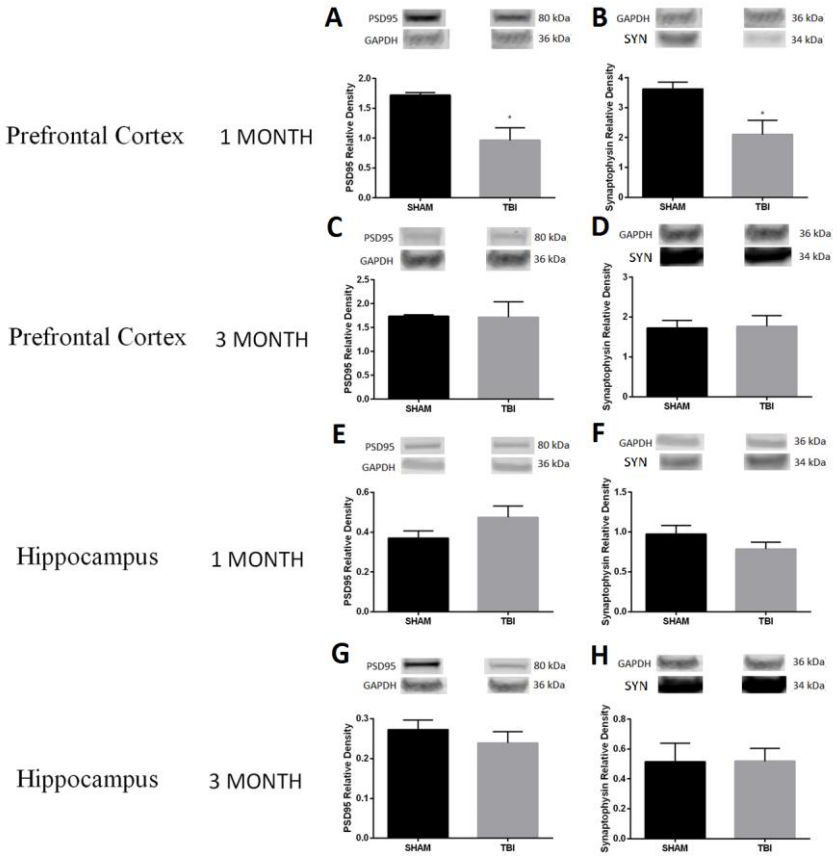

Figure 6: Synaptic structural damage was assessed by post-synaptic density 95 (PSD-95) and synaptophysin markers. Western blot images of PSD-95 and synaptophysin markers as well as GAPDH (housekeeper protein) at the A-D) PFC and E-H) hippocampus for each of the time point. The graphs illustrate the relative density of A,C,E,G) PSD-95 and B,D,F,H) synaptophysin in TBI animals when compared to sham in the PFC at A-B) 1 month and C-D) 3 months post-injury, and in the hippocampus at E-F) 1 month and G-H) 3 months post-injury. Graph represent the mean ± SEM, (n=5 per group; *p<0.05 compared to shams).

Assessment of axonal integrity with NF-L found no significant differences within the PFC (1.393±0.082 vs 1.405±0.059 in shams, *t*(8)=0.123, p=0.905) or the hippocampus (1.009±0.076 vs 1.113±0.069 in shams, *t*(8)=1.019, p=0.338) at 1 month-post-injury; however, a trend towards a decrease in the hippocampus at 3 months post-injury was observed (0.88±0.107 vs 1.184±0.071 in shams; *t*(6)= 2.38, p=0.06) (Fig. 7D). In contrast, a significant increase in levels of NF-H was seen at 1 month post-injury within the PFC (1.398±0.11 vs 0.922±0.138 in shams;

t(8)= 2.70, p=0.027), which had resolved by 3 months post-injury (0.838±0.148 vs 1.216±0.234 in shams, *t*(8)=1.365, p=0.209). No changes in NF-H were noted within the hippocampus at 1 month (1.459±0.213 vs 1.5±0.101 in shams, *t*(8)=0.177, p=0.864) or at 3 months (2.352±0.427 vs 2.642±0.204 in shams, *t*(7)=0.561, p=0.593) post injury. Integrity of myelin was evaluated with MBP, with a trend towards an increase in the PFC at 1 month post-injury (0.635±0.068 vs 0.404±0.074; *t*(7)= 2.304, p=0.055) which had resolved by 3 months post-injury (1.006±0.018 vs 0.966±0.07 in shams, *t*(8)=0.54, p=0.604) (Fig. 7I & K). No differences in MBP were seen at 1 month (1.121±0.151 vs 0.915±0.109 in shams, *t*(6)=1.102, p=0.313) or 3 months (0.494±0.062 vs 0.571±0.103 in shams, *t*(7)=0.595, p=0.571) post-injury in the hippocampus.

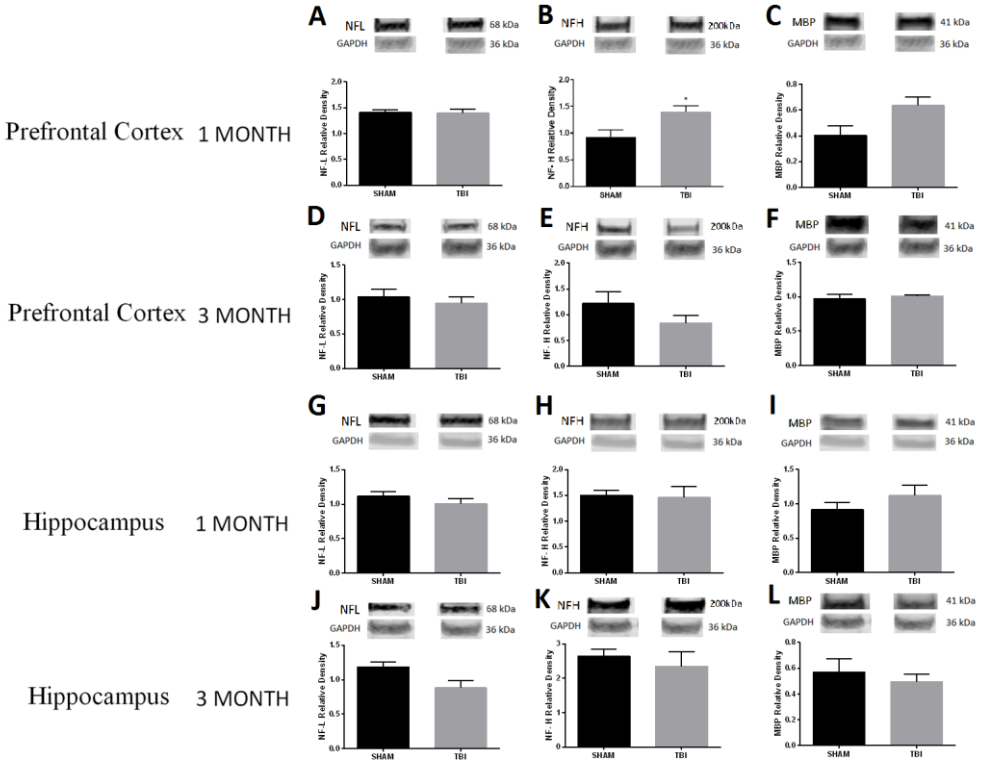

Figure 7: Neuronal structural damage was assessed by neurofilament-light chain (NF-L), neurofilament-heavy chain (NF-H) and myelin basic protein (MBP) markers. Western blot images of NF-L, NF-H and MBP markers as well as GAPDH (housekeeper protein) in the A-F) PFC and G-L) hippocampus for each of the time point. The graphs illustrate the relative density of NF-L, NF-H and MBP in TBI animals when compared to sham in the PFC at A-C) 1 month and D-F) 3 months post-injury, and in the hippocampus at G-I) 1 month and J-L) 3 months post-injury. Graph represent the mean ± SEM, (n=5; *p<0.05 compared to shams).

2.8 *Oxidative stress*

Oxidative stress was assessed by evaluating levels of the antioxidant, SOD-1 (superoxide dismutase 1) (Fig. 8). In the PFC, there was a significant increase in the relative density of SOD-1 at 1 month post-injury (1.082±0.033 vs 0.85±0.032 in shams, $t(6)= 5.074$, p=0.002), which had resolved by 3 months (0.67±0.102 vs 0.602±0.049 in shams, $t(7)=0.547$, p=0.602) post-injury. In the hippocampus, no changes in SOD-1 were noted at either time-point; 1 month (0.934±0.043 vs 0.881±0.052 in shams, $t(8)=0.787$, p=0.454), 3 months (0.679±0.056 vs 1.09±0.203 in shams, $t(7)=1.764$, p=0.121).

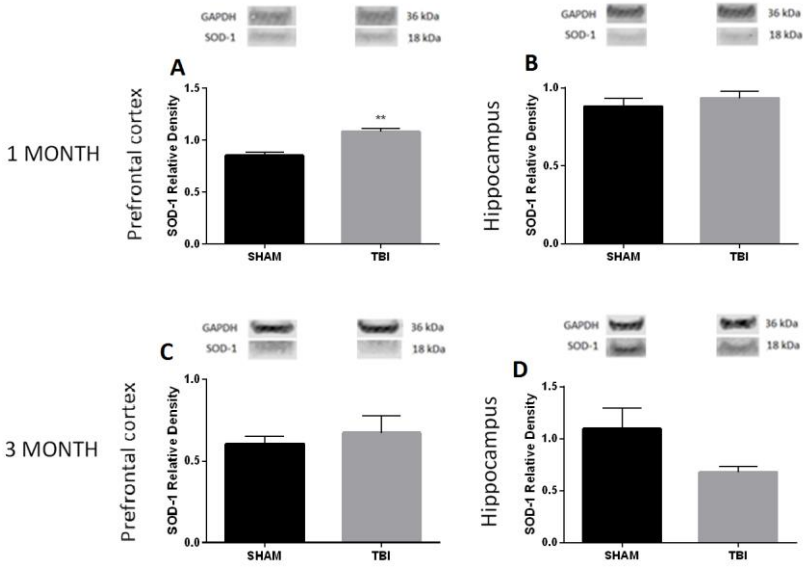

Figure 8: Oxidative stress was assessed by superoxide-dismutase 1 (SOD-1) marker. Western blot images of SOD-1 and GAPDH (housekeeper protein) in the A,C) PFC and B,D) hippocampus for each of the time points. The graphs illustrate the relative density of SOD-1 in TBI animals when compared to sham in the PFC at A) 1 month and C) 3 months post-injury, and in the hippocampus at B) 1 month and D) 3 months post-injury. Graphs represent the mean ± SEM, (n=5 per group; **p<0.01 compared to shams).

Table 1. Summary of behavioural results; changes in TBI when compared to shams at 1 month and 3 months post injury.

Test Paradigm	Behaviour measurement	1 month	3 month
Open Field Test (OFT)	Distance Travelled (m)	(Δ= -2.99)	↓ (Δ= -11.83)* p= 0.021
	Time in Center (s)	(Δ= 1.97)	(Δ= -1.64)
Elevated Plus Maze (EPM)	Time in open arms (s)	(Δ= 8.29)	(Δ= -15.17)
Forced Swim Test (FST)	Time Immobile (s)	↑(Δ=26.57)* p= 0.027	↑(Δ= 34.45)** p= 0.004
Y-Maze	Novel Preference	(Δ= -0.045)	(Δ= -0.018)
Barnes Maze	Escape Latency to box on Acquisition Training (s)	NA	Day 1: (Δ= -0.523) Day 2: (Δ= -15.80) Day 3: (Δ= 3.697)
	Escape Latency to Old box (s)	NA	(Δ= -13.78)
	Escape Latency to New Box (s)	NA	Trial 1: (Δ= -38.95)* p= 0.014 Trial 2: (Δ= -8.781)

Note: *p<0.05, **p<0.01, ↓= decrease in value when compared to shams, ↑= increase in value when compared to shams, Δ= (mean of TBI – mean of sham)

Table 2. Summary of histopathological results; changes in TBI when compared to shams at 1 month and 3 months post injury, at the prefrontal cortex and hippocampus region.

Markers	**Prefrontal Cortex**		**Hippocampus**	
	1 month	3 month	1 month	3 month
GFAP (glial fibrillary acidic protein)	↓ (Δ= -57.60)***** p= 0.019	(Δ= 0.59)	(Δ= -14.50)	(Δ= -8.17)
IBA1 (ionized calcium binding adaptor molecule 1)	↓ (Δ= -20.70)***** p= 0.049	(Δ= 6.15)	(Δ= -20.20)	(Δ= 19.06)
PSD-95 (postsynaptic density protein 95)	↓ (Δ= -0.76)***** p= 0.039	(Δ= -0.02)	(Δ= 0.11)	(Δ= -0.03)
Synaptophysin	↓ (Δ= -1.52)***** p= 0.049	(Δ= 0.05)	(Δ= -0.19)	(Δ= 0.003)
NF-L (neurofilament light chain)	(Δ= -0.01)	(Δ= -0.09)	(Δ= -0.10)	↓ (Δ= -0.30) p= 0.06
NF-H (neurofilament heavy chain)	↑ (Δ= 0.48)***** p= 0.027)	(Δ= -0.38)	(Δ= -0.04)	(Δ= -0.29)
MBP (myelin basic protein)	↑ (Δ= 0.23) p=0.055	(Δ= 0.04)	(Δ= 0.21)	(Δ= -0.08)
SOD-1 (superoxide dismutase 1)	↑ (Δ= 0.23)** p=0.002	(Δ= 0.07)	(Δ= 0.05)	(Δ= -0.42)

Note: *p<0.05, **p<0.01, ↓= decrease in value when compared to shams, ↑= increase in value when compared to shams, Δ= (mean of TBI – mean of sham)

3. Discussion

The current study investigated the effect of moderate-severe TBI on chronic changes in axonal and synaptic integrity, neuroinflammation and persistent functional deficits at 1 and 3 months post-injury. It was found that, following TBI, animals showed persistent depressive-like behaviour with increased time spent immobile in the FST at 1 and 3 months post-injury. A decrease in cognitive flexibility on the Barnes Maze was seen at 3 months post-injury, but no impairment was noted in learning and memory during the acquisition phase of the task nor in recognition memory on the Y-Maze (Table 1). Within the PFC, synaptic loss was noted at 1 month post-injury, as indicated by decreased levels of synaptophysin and PSD-95, which corresponded to a concomitant decrease in the number of astrocytes and microglia. Furthermore, other neuronal changes such as increases in NF-H and MBP, were also observed at this early timepoint in the PFC. These changes were resolved by 3 months post-injury. In contrast, within the hippocampus, no changes in the number of inflammatory cells was noted at either time-point nor any effect on synaptic integrity, with the main finding a decrease in relative expression of NF-L at 3 months post-injury.

The most notable functional finding was that TBI led to the development of persistent depressive-like behaviour that had not resolved by 3 months post-injury (Table 1). Although no ongoing motor impairment was noted on the rotarod, with performance at sham level at 3 weeks post-injury, there was a decrease in locomotor activity at 3 months post-injury on the open field. This may relate to lack of motivation to explore the open field (Correia et al., 2017), but further studies will be needed to confirm this theory. Nonetheless, it appears that the increase in immobility time in the FST reflects a behavioural response, rather than gross motor impairment. This increase in immobility time is thought to be indicative of behavioural despair and, given that it decreases with administration of antidepressants (Castagné V et al., 2009), is thought to provide an indicator of depressive-like behaviour. The observations in this study are in line with clinical studies, which have reported the prevalence of depression in TBI patients to be as high as 77% (Osborn et al., 2014b), with 30-40% of individuals suffering from major depressive

disorder within a year post-injury (Jorge et al., 2004). In contrast, pre-clinical studies have had mixed results, with reports of no difference in behaviour on the FST at 1 month post moderate controlled cortical impact (Geppetti et al.; Tucker et al., 2017; Wang et al., 2011) or 6 months post-lateral fluid percussion injury (Jones et al., 2008). Conversely, Milman *et al* and Taylor *et al* found increased immobility at 2-3 months post-injury utilising a diffuse weight drop model and a more severe CCI model, respectively (Milman et al., 2005; Taylor et al., 2006). This suggests that, in order for depressive-like behaviour to be present at sub-acute-chronic time-points post-injury, a wider spread injury may be required, like the diffuse model of injury employed here.

Indeed, within this study, the profile of deficits, in depressive-like behaviour and reduced cognitive flexibility, align with structural changes that were mostly noted within the PFC and not the hippocampus (Table 2). The PFC plays a central role in emotional regulation, with reductions in PFC volume following TBI associated with the development of depressive symptoms post-TBI (Hudak et al., 2011; Jorge et al., 2004; Rao et al., 2010). In regards to cognitive flexibility, lesions within the PFC lead to an impairment in the ability to modify a response in relation to new information of a learned task (Bizon et al., 2012; Rabinowitz and Levin, 2014), similar to the deficit seen here, with post-TBI animals taking longer to locate the escape box when it was moved during the probe trial. These deficits were associated with decreased levels of PSD-95 and synaptophysin within the PFC, suggesting synaptic dysfunction. Few studies have examined the effect of TBI on synaptic morphology in the PFC region post-TBI, with Hoskinson *et al* finding alterations in dendritic spine density at 4 months following a parietal CCI injury (Hoskison et al., 2009) and Zhao *et al* finding a significant reduction of dendritic spine density in layer II/III pyramidal neurons of the medial PFC at two weeks post-FPI (Zhao et al., 2017). This supports the idea that TBI can cause significant disruption to the PFC region. Notably, although PSD-95 and synaptophysin had returned to sham levels by 3 months post-injury, functional deficits persisted, suggesting that there may have been persistent alterations in the circuitry (ex: serotonin circuitry, receptor expression) of the PFC. Specific

examination of synaptic morphology within different layers and specific regions of the PFC may provide further insight into these alterations. It might also be beneficial to investigate total neuron number in future studies, for a more precise measurement of synapse loss.

As well as evidence of synaptic disruption, levels of NF-H were also significantly increased at 1 month post-injury within the PFC, before returning to baseline at 3 months, although no changes were noted in levels of NF-L. Neurofilaments are the dominant intermediate filament of axons (Siedler et al., 2014; Yuan et al., 2012) and are thought to be a key contributor to axon strength and resilience to mechanical stretch (Hill et al., 2016). Immediately following diffuse impact acceleration and fluid percussion injuries, neurofilament compaction due to side-arm phosphorylation or proteolysis is known to be a key indicator of axonal integrity (Okonkwo et al., 1998; Povlishock et al., 1997). Activation of neuronal proteases is also associated with an acute reduction in levels of neurofilament as measured via western blot encompassing the light, medium and heavy subtypes (Posmantur et al., 1994; Serbest et al., 2007). The increase in NF-H at one month post-injury may therefore reflect a rebound reparative response following this acute injury phase involving disruption and loss of these proteins. Another potential explanation for the increase seen in NF-H in the current study is as a protective mechanism against toxic oxygen radical species. Wataya *et al* found that NF-H may act to sequester toxic lipid peroxidation byproducts in aldehydes, in order to protect critical active sites on proteins from oxidative attack (Wataya et al., 2002). NF-H is thought to preferentially perform this task as it is a lysine-rich protein, the component providing the buffering mechanism (Bogdanova et al., 2013). Unfortunately, within our study, we did not investigate oxidative stress markers directly, but instead used a measurement of superoxide dismutase 1 (SOD1), an antioxidant enzyme against superoxide radicals (Ansari et al., 2008). Levels of SOD1 were elevated, like those of NF-H, at 1 month post-injury within the PFC, suggesting that this could be a similar protective mechanism against elevated levels of reactive oxygen species (ROS). Indeed, overexpression of SOD1 is known to be neuroprotective in a number of models of brain injury (Endo et al., 2007; Sugawara et al., 2002). Previous studies have shown ongoing oxidative stress within the

injured parietal cortex at 1 month following FPI injury, as indicated by an increase in levels of oxidative damaged lipids and proteins (Lima et al., 2008; Silva et al., 2011). Future studies should confirm whether there is evidence of ongoing oxidative stress within the PFC following a purely diffuse weight drop injury.

Surprisingly, despite the pattern of behavioural deficits seen here and the evidence of synaptic dysfunction, increased neuroinflammation was not seen in the PFC at either 1 or 3 months post-injury. In fact, a reduction in the number of microglia and astrocytes was noted in this region at 1 month post-injury. Given that these cells have a number of beneficial functions, including release of neurotrophic factors, such as BDNF (Gomes et al., 2013; Toyomoto et al., 2005), modulation of neurotransmitter levels within the synapse (von Blankenfeld and Kettenmann, 1991) and supply of energy to neurons (Belanger and Magistretti, 2009), this decrease may not be beneficial. Indeed, previous reports have found a decrease in levels of GFAP, a cytoskeletal protein expressed by many astrocytes, in the PFC of depressed patients (Johnston-Wilson et al., 2000; Miguel-Hidalgo, 2005; Turner et al., 2004). It has been proposed that this alteration in astrocytes may influence glutamatergic signalling, thereby contributing to pathology (Chung et al., 2015; Medina et al., 2016). The mechanism driving this decrease in resident immune cell numbers within the PFC at 1 month post-injury is not known, but it is possible that these cells may have migrated to other sites, such as the corpus callosum (Plummer et al., 2018), with restoration of numbers by 3 months post-injury. Further studies are needed to confirm this result. Furthermore, as only total number of microglia were assessed, it is important to also confirm whether they are resting or reactive to provide a clearer picture of the neuroinflammatory reaction after injury. Besides glial cells, neuroinflammation can also be evaluated by neurochemical changes as Lozano *et al.* has shown the mobilization of chemokines and cytokines to the site of injury post-TBI which may be neuroprotective or neuro-damaging depending on time after injury (Lozano et al., 2015). The relationship between TBI and neurochemical changes should be investigated in future studies to provide a more complete neuroinflammatory role post-TBI.

In contrast to the evidence of structural changes within the PFC at subacute time points post-injury, this study found minimal pathology within the hippocampus. This lack of hippocampal pathology is supported by the lack of deficits in the learning phase of the Barnes Maze or in recognition memory as assessed by the Y-Maze. These tasks preferentially assess hippocampal dependent learning with Conrad *et al* demonstrating that bilateral damage to the CA3, CA4 or dentate gyrus led to a decrease in spatial memory on the Y Maze (Conrad et al., 1996). In regards to the lack of hippocampal mediated cognitive impairment seen in this study, previous studies, contrastingly, have shown persistent cognitive deficits post-TBI, with, for example, Pearce et al observing significant deficits in spatial learning ability in the MWM beginning at two months and lasting up to one year following lateral FP brain injury (Pierce et al., 1998), with similar reports of cognitive deficits from one month to one year following CCI injury (Shear et al., 2004). This most likely relates to the more significant hippocampal damage associated with these injury models, with CCI associated with a 60% loss of hippocampal synapses acutely, that had still not recovered to pre-injury levels by day 60 (Scheff et al., 2005). Similar levels of significant hippocampal cell death have been reported following FPI (Royo et al., 2006), unlike the lack of synaptic damage seen here at either one or three months post-injury. Previous studies utilising the diffuse impact-acceleration model have similarly reported a lack of hippocampal dependent cognitive deficits on the MWM or radial arm maze (Hallam et al., 2004; Maughan et al., 2000), with a corresponding lack of neuronal loss within this area (Hallam et al., 2004).

In conclusion, this study found that the PFC is significantly affected at one month following a diffuse TBI. There was evidence suggestive of both synaptic and axonal disruption that were associated with a decrease in the number of astrocytes and microglia. These alterations within the PFC also coincide with the impairments on the FST and decreased cognitive flexibility seen after injury. In contrast, the hippocampus was relatively spared at 1 and 3 months post-injury, with future studies needing to examine later time-points to determine if hippocampal damage remerges. Nevertheless, our study provides evidence of early structural changes in the

prefrontal cortex after moderate-severe diffuse TBI, which, although resolved at sub-chronic stages, may contribute to long-term deficits as a result of neuropathological re-emergence or progression at chronic stages in other brain areas and thus may be linked to early neurodegeneration.

4. Experimental Procedure:

4.1 *Animals*

Adult male Sprague-Dawley rats (10-12 weeks) (were used under approval of the University of Adelaide Animal Ethics Committee (M-2015-027). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery or moderate, diffuse TBI, with one subset subject to a functional assessment battery at 1 month post-injury (shams n=14; TBI n=19) and another at 3 months post-injury (shams n=13, TBI n=14). Following completion of functional assessment, animals were perfused and the brains collected for either histological or molecular analysis.

4.2 *Injury Model*

The Marmarou impact-acceleration model (Marmarou et al., 1994) was utilized, as it has been extensively validated as a model of diffuse injury (Xiong et al., 2013b). Animal weights ranged from 350-380g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. They were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout. A midline incision was made to facilitate the placement of a metal disc centrally between lambda and bregma. Animals assigned to undergo TBI were then transiently taken off ventilation, strapped onto a foam, with injury induced by releasing a 450g weight from a height of 2 metres down a clear tube onto the centre of the metal helmet. Contact was observed to ensure single, direct impact without a rebound hit. Animals were then subject to hypoxic conditions (2L/min nitrogen; 0.2L/min oxygen) for 10 minutes, to replicate the clinical effects seen following this injury model without

ventilation, as this hypoxic condition is known to exacerbate the severity of the injury (Hellewell et al., 2010; Ishige et al., 1987a). Hypoxia alone had similar levels of cytoskeletal structure and neuroinflammation as shams under normoxic ventilation (as in this study) as reported by Hellewell *et al.* previously (Hellewell et al., 2010). Wound closure was performed with surgical staples. Successful induction of moderate to severe TBI was assessed 24 hours later by rotarod scores of below 100, weight reduction of 5-10% and clinical signs (paresis and hunched posture). Animals in the moderate to severe TBI group that were not meeting the above criteria were excluded from the study. Shams assessed at the same timepoint (24 hours) exhibited none of the clinical signs and had rotarod scores of more than 100.

4.3 *Functional studies*

Functional tests assessing cognition, anxiety, depression and motor function were performed at 1 month and 3 months post-injury. All functional data was recorded using the ANY-maze Video Tracking System version 4.99m (Stoelting Co.). The functional tests were done in order from least to most aversive (stress inducing) except the rotarod test which was done at specific timepoints throughout the experiment regardless of other tests.

4.3.1 Rotarod

The rotarod is used as a standard motor coordination evaluation test for rodents (Deacon, 2013). Animals were placed on an elevated horizontal rod that rotates along the longitudinal axis. Animals were first habituated on the stationary rod for 10 secs. Then, for every 10 sec thereafter, the rotation of the rod was accelerated at a constant rate of 3rpm until the 100 sec mark (maximum acceleration speed of 30rpm). Animals were kept on the rotarod at the maximum speed for a further 20 secs before decelerating the speed and removing the animal from the test. The rotarod score was measured by the latency of the animal to fall off the rod. Animals were trained for 3 consecutive days or until a score of 120 (baseline) was achieved. Following injury, animals were tested on the rotarod at 24 hours then every 7 days following, i.e., day 8, day 15 and so on till the endpoint of the study.

4.3.2 Open Field Test

The open field test (OFT) is a common tests of locomotor activity (Malkesman et al., 2013). Animals were placed in the centre of a large square box (95cm x 95 cm) with walls at height 44.5cm and the total distance travelled over a 5 minute period was recorded.

4.3.3 Elevated Plus Maze

The elevated plus maze (EPM) is widely used in anxiety research (Malkesman et al., 2013). Animals were placed in the centre of an elevated (50cm in height) cross-shaped maze consisting of two open and two closed (walls of height 40cm) maze arms (each of length 50cm), facing the open arms, for 5 minutes. Time spent in the closed arms versus open arms was recorded, with increased time spent in the closed arms thought to represent anxiety-like behaviour.

4.3.4 Y-Maze

The Y-Maze is used to test cognition in terms of spatial recognition memory (Wright and Conrad, 2005). In the Y-Maze, animals are placed in an equal angled Y-shaped arena, with each arm of the maze identical in size and shape but visually distinct (due to cues on the wall) from the others. The test involves two 3-minute trials separated by 1 hour. In the first trial, one arm was closed off with a clear wall (novel arm) to enable the animal to visually recognise its presence; in the second trial, this novel arm became accessible (wall removed). In cases of reduced spatial reference memory, the animal spends less time within the novel arm.

4.3.5 Barnes Maze

The Barnes maze evaluates spatial learning and memory in rats (Sunyer et al., 2007). The maze is an elevated, open circular black platform with 18 holes evenly distributed along its edges. One of the holes is pre-determined as the escape hole with a black escape box placed below the hole. The Barnes maze test was preformed over the course of five days; three days of acquisition trials, a rest day (no interaction with the animals) and a probe day. During the acquisition days, animals were subject to two trials spaced 15 mins apart. They were placed in the centre of the Barnes maze in a brightly lit room with the time taken for the animal to find and enter the escape box recorded. On day 5, the escape box was relocated to a new hole and two trials conducted 1 hr apart. In trial 1, the time taken for the animal to reach the old position of the escape hole was

recorded. In both trials, the time taken to locate and enter the newly relocated escape box was recorded as a measure of cognitive flexibility.

4.3.6 Forced Swim Test

The forced swim test (FST) is widely utilised to assess depressive-like behaviour (Bogdanova et al., 2013). The animal was placed within an inescapable glass cylinder filled halfway with 25 °C water, adjusted for the animal's length so that the hind legs does not touch the bottom of the cylinder, for 5 minutes. The time spent immobile was recorded as a measure of behavioural despair.

4.4 *Tissue Collection and Processing*

Animals were randomly assigned for further processing, either by molecular analysis or immunohistochemistry, during euthanasia. Animals that were to be used for molecular analysis were transcardially perfused with 0.9% saline and the brain dissected with the prefrontal cortex (PFC) and hippocampus taken (n=5 per group). Samples were snap-frozen in liquid nitrogen before being stored at -80°C. The samples were then homogenised via sonication in freshly prepared buffer (20mM Tris-HCl pH 7.5, 2mM EDTA, 0.5mM EGTA, 140mM 2-mercaptoethanol) with protease inhibitor cocktail (Sigma), 10uL/mL aprotinin, leupeptin, pepstatin A and 10mM PMSF. Each sample underwent 3 bursts of 10 seconds duration under a sonicator probe. Homogenised samples were centrifuged for 30 minutes at 14000 rpm and 4°C, before supernatant was collected. Protein concentration was estimated with Pierce BCA Protein Assay (ThermoScientific) at 750nm absorbance.

Animals that were to be used for immunohistochemical analysis were transcardially perfused with 0.2mL heparin + 10% formalin. Brains were removed and post-fixed in 10% formalin for 24 hours, then blocked into 2mm coronal sections and embedded in paraffin-wax. To examine the PFC, three consecutive 5μm coronal slices were taken beginning at +4.20mm from Bregma for each animal. For hippocampal sections, three serial 5μm coronal slices per animal were taken starting at -1.60mm representing anterior hippocampus, -2.80mm representing mid

hippocampus and at -3.80mm representing posterior hippocampus. Tissue mounted slides were allowed to dry at 37°C overnight.

4.5 *Western Blot*

Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels (Life Technologies) with 50ug of protein loaded per well. Gels were run at 150V for 30-45 minutes, depending on the molecular weight of the protein of interest, and transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes x 5 minutes), stained with Ponceau S red solution (Fluka Analytical) (5 minutes) for protein visualisation, and washed with distilled water until removal of Ponceau had been achieved.

Membranes were incubated for 2.5 hours with primary and secondary antibodies in 1X iBind solution using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations: mouse anti-post-synaptic density protein 95 (PSD-95) (1:1000, ab2723 or ab18258, Abcam), rabbit anti-synaptophysin (1:1000, ab32127, Abcam), mouse anti-myelin basic protein (MBP) (1:250, ab62631, Abcam), mouse anti-neurofilament (1:300, ab24574, Abcam), rabbit anti-superoxide dismutase 1 (SOD1) (1:1000, ab13498, Abcam), and the primary housekeeping antibody chicken anti-GAPDH (1:4000, ab83956, Abcam). Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. Western blots were imaged using an Odyssey Infrared Imaging System (model 9120; software version 3.0.21) (LI-COR, Inc.) at a resolution of 169μm. Semi-quantitative analysis of band signals was performed using ImageJ version 1.49 and Image Studio Lite version 5.2. Normalization of blot runs at 1 month and 3 month were performed using a single control sample of the respective time points. Thus, relative density of the samples were calculated based on the adjusted density for each blot, as below:

Adjusted density = $\frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$

Relative density = $\frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$

4.6 *Immunohistochemistry*

Immunohistochemistry (IHC) was performed as per standard procedure. In brief, slides were oven-dried, de-waxed in xylene, rehydrated in ethanol and then placed into methanol with 0.5% hydrogen peroxide to block endogenous peroxidases. Then the slides were washed twice in phosphate buffered saline (PBS) and were blocked in normal horse serum (NHS) (1:30) for 30 minutes before incubation overnight with primary antibody (Table 3). The following day, slides were washed twice in PBS before application of secondary antibody (DAKO, 1:250, 30 minutes). Slides were once again washed twice with PBS, and then incubated with streptavidin peroxidase conjugate (SPC) (1:1000, 60 minutes). Slides were given a final wash in PBS, then incubated with 3,3'-Diaminobenzidinetetrahydrochloride (Njoku et al.) (1:50, 7 minutes) for antigen retrieval. Lastly, slides were counterstained with haematoxylin, placed in ethanol and subsequently in xylene, before mounting on cover slips.

Following staining, sections were scanned with Nanozoomer slide-scanner (Hamamatsu, Japan) and images viewed on NDPview (version 2). GFAP Iba1 immunoreactivity was assessed quantitatively by counting the reactive and immunopositive cells per mm^2 within the hippocampus (CA1+ CA3+DG region) and PFC (prelimbic region). The experimenter was blinded to the experimental group during cell counting and counts were performed twice.

Table 3. Primary antibodies investigated using immunohistochemistry.

Primary Antibody	**Analysis Target**	**Antigen Retrieval**	**Host animal and Dilution**	**Manufacturer**
GFAP	Astrocyte reactivity	Citrate	Rabbit 1: 40,000	DAKO
Iba1	Microglial reactivity	Citrate	Rabbit 1: 20,000	Wako

[GFAP: Glial Fibrillary Acidic Protein, Iba1: Ionized calcium Binding Adaptor molecule 1]

4.7 *Statistics*

Except where outlined below, all data was analysed via two-tailed unpaired t-test using GraphPad Prism software. A repeated two-way analysis of variance was performed on the rotarod scores and on the acquisition days of the Barnes maze test. P values <0.05 were considered statistically significant.

Chapter 3

Cognitive and neuropsychiatric impairments vary as a function of injury severity at 12 months post-experimental diffuse traumatic brain injury: Implications for dementia development

Statement of Authorship

Title of Paper	Cognitive and neuropsychiatric impairments vary as a function of injury severity at 12 months post-experimental diffuse traumatic brain injury: Implications for dementia development.
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Name of Principal Author (Candidate)	Alina Arulsamy		
Contribution to the Paper	Performed analysis of behavioural data, interpreted data and wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	8/03/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript editing		
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Cognitive and neuropsychiatric impairments vary as a function of injury severity at 12 months post-experimental diffuse traumatic brain injury: Implications for dementia development.

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AA was involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

Abstract

Traumatic brain injury (TBI) is a common risk factor for later neurodegeneration, which can manifest as dementia. Despite this, little is known about the time-course of development of functional deficits, particularly cognitive and neuropsychiatric impairments, and whether these differ depending on the nature of the initiating insult. Therefore, this study investigated long term functional impairment at 12 months post-injury following diffuse TBI of different severities. Male Sprague-Dawley rats (420-480g; 10-12 weeks) were either given a sham surgery (n=14) or subjected to Marmarou's impact acceleration model of diffuse TBI for a single mild TBI (n=12), repetitive mild TBI (3 mild diffuse injuries at 5 day intervals) (n=14) or moderate to severe TBI (n=14). At 12 months after injury, they were tested on a functional battery encompassing motor, neuropsychiatric (anxiety and depressive-like) and cognitive function. Our results showed that moderate to severe TBI animals exhibited significant impairments in cognitive flexibility (p=0.009) on the Barnes maze when compared to age-matched sham animals. Neither repetitive mild TBI nor single mild TBI animals showed significant functional impairments when compared to shams. Thus, this study provides the first insight into chronic functional impairments associated with different severities of diffuse TBI, with moderate to severe TBI being a higher risk factor for impaired cognitive function at 12 months post-injury. Taken together, this may have implications for risk of dementia development following different severities of injury.

Keywords: cognition, anxiety, diffuse injury, injury severity, chronic outcomes

1. Introduction

Traumatic brain injury (TBI) covers a broad spectrum of disease, ranging from milder concussive insults to severe injuries. Since the first documentation of TBI leading to the development of parkinsonian-like symptoms in professional boxers (Martland, 1928b), the research community has regarded TBI as not just a single insult, but as an injury that has ongoing functional consequences (Masel and DeWitt, 2010b). Repetitive mild (rmTBI) is linked with the later incidence of depression (Jorge et al., 2004; Vargas et al., 2015a), anxiety (Max et al., 2011) and impairments in learning and memory (Guskiewicz et al., 2005; McAllister et al., 2012). Studies on contact-sport athletes have associated a history of multiple concussions to a range of behavioural abnormalities, memory deficits and even parkinsonism (McKee et al., 2009) in later years. Conversely, following a single severe injury, cognitive impairments are most notable, with emergence of deficits in different cognitive domains over time, even in the subacute phase (Till et al., 2008). For example, serial neuropsychological testing over 5 years following injury found that 30% of patients who had experienced a moderate/severe TBI had clinically significant decline in two or more domains of cognitive functioning (Till et al., 2008). Higher rates of anxiety and depression are also reported chronically following a single moderate/severe injury, with reports of clinically significant depression in 46% of individuals at 10 years post-injury (Draper et al., 2007), compared to ~20% in the general population (Bromet et al., 2011).

Experimental models of TBI also support the persistence of functional deficits following injury (McAteer et al., 2016; Mouzon et al., 2018; Petraglia et al., 2014). Animals subjected to a focal TBI induced by the controlled cortical impact model demonstrated persistent subtle cognitive deficits on the Morris Water Maze at 12 months post injury (Dixon et al., 1999). Similarly, animals injured via fluid percussion (FPI), which produces a mixed focal and diffuse injury, also had persistent cognitive deficits at 12 months post-injury (Hausser et al., 2018; Sell et al., 2017). Following purely diffuse axonal injury (Lv et al.), cognitive deficits, evidenced

by impaired spatial and recognition memory on the Barnes Maze, as well as increased anxiety, have been reported at 3 months post-rmTBI (McAteer et al., 2016), while impaired spatial learning and cognitive flexibility and increased depressive-like behaviour were observed at 3 months following a single moderate/severe diffuse TBI (Arulsamy et al., 2018b). To date, however, the behavioural effects of a purely diffuse injury have not been investigated pre-clinically beyond this 3 month time-point. This represents a significant gap in the existing literature, as "pure" forms of focal injury occur in only 28% of moderate-severe TBI cases, while diffuse axonal injury is seen in 72% of individuals, with "pure" diffuse axonal associated with significantly lower scores on the Glasgow Coma Scale (Skandsen et al., 2010).

These persistent functional impairments seen following injury may set the stage for later pathology, including a significantly increased risk for the development of neurodegenerative diseases, such as AD (Fleminger et al., 2003a; Nemetz et al., 1999a; Plassman et al., 2000), Parkinson's (PD) (Bower et al., 2003; Gardner et al., 2015a; Goldman et al., 2006), chronic traumatic encephalopathy (CTE) (McKee et al., 2009; Omalu et al., 2011; Omalu et al., 2005), fronto-temporal dementia (FTD) (Deutsch et al., 2015a; Rosso et al., 2003) and motor neurone disease (MND) (Chen et al., 2007b; Chio et al., 2005), as reviewed in (Faden and Loane, 2015; Li et al., 2017; McKee and Daneshvar, 2015; Sundman et al., 2014a). Of these, the link between TBI and the later emergence of dementia has received the most attention to date. A dose-response relationship is thought to exist in terms of the risk of developing neurodegenerative disease (Plassman et al., 2000), with more severe injury associated with greater risk, but even a single mild TBI may be linked to an increased risk of dementia (Lee et al., 2013). A retrospective study utilizing health data from emergency department visits showed an increased risk of dementia with a minimum hazard ratio of 1.46 in moderate to severe TBI patients and a minimum hazard ratio of 1.1 in mild TBI patients, over a 5-7 year follow-up period (Gardner et al., 2014b). A similar risk was reported in a Taiwan-based retrospective cohort study, in which individuals who had experienced a moderate to severe TBI showed a 1.68 fold higher risk of dementia than non-TBI patients (Wang et al., 2012).

Interestingly, the type of dementia that develops may differ depending on the nature of the initiating insult. For example, it is hypothesised based on case studies that a single moderate/severe TBI may be more strongly associated with accelerating age-related dementia, such as AD (Fleminger et al., 2003a; Mortimer et al., 1991), while rm TBI may be more strongly linked to CTE (McKee et al., 2009). However, the neuropathology following both single injury and rmTBI shares similarities, including the accumulation of hyperphosphorylated tau in the form of neurofibrillary tangles (NFTs) at the base of the sulci (McKee et al., 2013a; Stein et al., 2014) and the development of a persistent inflammatory response post-injury (Aungst et al., 2014; McKee and Daneshvar, 2015; Turner et al., 2016). Indeed, it has been suggested that TBI induced neurodegeneration may be its own unique entity, with further research needed into this question. Thus, this study aimed to document the range of chronic functional impairments, including in motor function, neuropsychiatric function and cognition, that may be associated with the different TBI severities (mild TBI, rmTBI TBI and moderate/severe TBI) at 12-months post injury in an experimental model of DAI. Furthermore, the study assessed whether functional changes at 12-months post-injury were associated with alterations in either neuronal number or integrity in the prefrontal cortex (PFC), a key region for cognitive function.

2. Results

2.1. *Locomotion assessment*

General locomotor activity was assessed as distance travelled (m) in the OFT. A one-way ANOVA showed a significant main group effect in the distance travelled in the OFT ($F_{3,50}$ = 3.234, p=0.030). However, post-hoc analysis showed no significant changes (p>0.05) in distance travelled when the TBI groups were compared to shams at 12 months post-injury in the OFT. Nevertheless, the single mild TBI group did show significantly higher locomotor activity when compared to repetitive mild TBI animals (29.45m ±3.07 vs 18.68m ±2.09, p= 0.021) (Fig 1A). This was confirmed by the generation of a heat-map showing activity within the OFT (Fig 1B), which shows much greater coverage of the apparatus in the single mild TBI

animals. A similar pattern in locomotor activity was seen in the distance travelled in the elevated plus maze, with a significant main group effect ($F_{3,50}$ = 4.963, p= 0.004) driven by the single mild TBI group having a significantly higher distance travelled when compared to both the repetitive mild TBI group (6.14m ±0.60 vs 3.69m ±0.32, p=0.006) and the moderate/severe TBI animals (6.14m ±0.60 vs 3.82m ±0.62, p=0.01) after post-hoc analysis (Fig 1C). Locomotor assessment in the Y-maze showed a non-significant main group effect ($F_{3,50}$ = 2.114, p= 0.110) (Fig 1D).

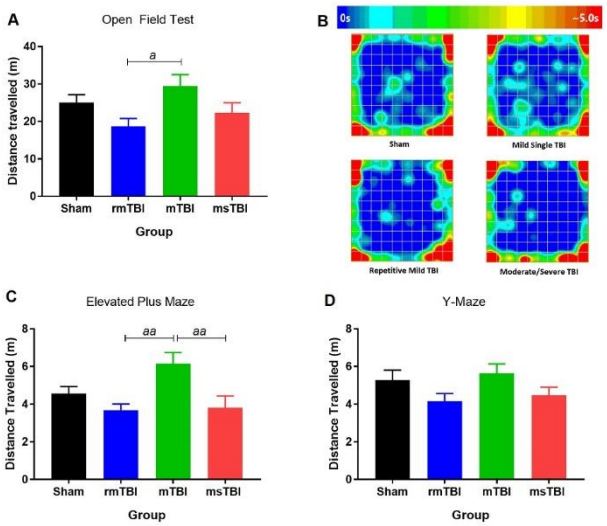

Figure 1: Distance travelled (m) as a measure of locomotion on A) open field maze, C) elevated plus maze and D) Y-maze post injury. B) Heat map analysis on open field maze indicating location and exploratory time within the open field post injury. Graphs represent the mean ± SEM, (n= 12-14per group; aa p<0.01,a p<0.05 compared between injury groups).Heat maps are from group composites.

2.2. *Anxiety-like behaviour*

Anxiety-like behaviour was assessed through various parameters in the open field and elevated plus mazes. Both assess different anxiety stimuli in the animal, with the open field assessing anxiety over open spaces and the elevated plus maze assessing anxiety over open spaces and height (Walf and Frye, 2007). In the open field, animals showed no significant main group

effect either in time spent rearing ($F_{3,50}$ = 2.17, p= 0.103) or time spent in the centre of the field ($F_{3,50}$ = 0.716, p= 0.547) (Fig 2A and 2B).

However, in the elevated plus maze, there was a significant main group effect in the time spent in the open arms ($F_{3,48}$ = 3.984, p= 0.013), as well as the number of open arm entries (H= 14.48, p= 0.002) and the number of crossings (H= 12.14, p= 0.007). The mild TBI group showed the least anxiety-like behaviour, with the most time spent in the open arms (95.3 secs ±17.44) and the highest number of both open arm entries 7.5 (5-14) and crossings 14 (9-28), when compared to other groups. These were not significant when compared to shams; time in open arm (95.3 secs ±17.44 vs 61.24 ±10.66 in shams, p= 0.248), number of open arm entries (7.5 (5-14) vs 6 (1-17) in shams, p= 0.944) and number of crossings (14 (9-28) vs 12 (2-31) in shams, p= 0.786), but was significant only when compared to moderate-severe TBI; time in open arms (95.3 secs ±17.44 vs 31.01 secs ±7.6, p=0.007) (Fig 2C), number of open arm entries (7.5 (5-14) vs 3 (0-10), p= 0.001) (Fig 2D) and number of crossings (14 (9-28) vs 6.5 (0-20), p= 0.006) (Fig 2E). Indeed this pattern can be seen in the heat-map, which shows the average amount of time spent in each part of the elevated plus maze across the injury groups, with the single mild TBI animals showing the highest amount of time in the open arms (Fig 2F).

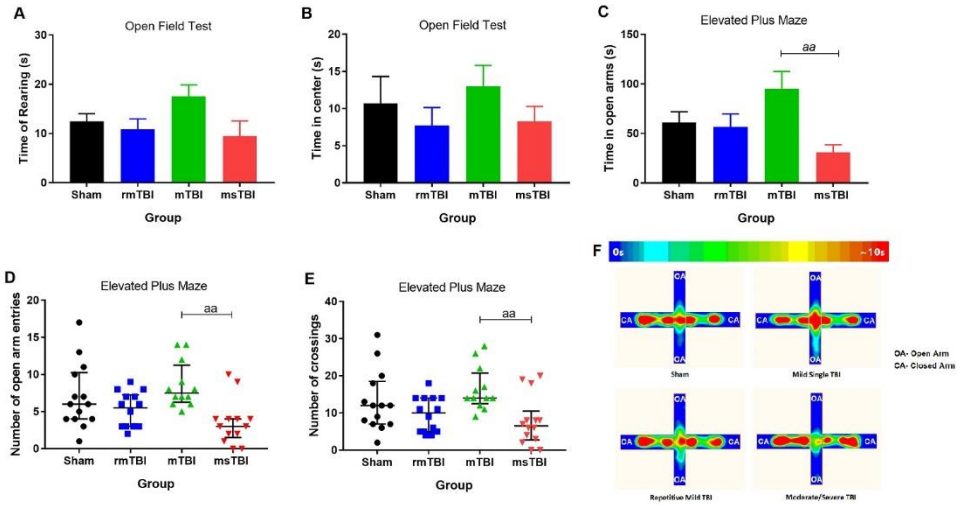

Figure 2: Anxiety-like phenotype as measured by A) time spent rearing(s) and B) time spent in the centre(s)of the open field as well as measured by C) time in the open arms(s), D) number of entries into the open arms and E) number of crossings in the elevated plus maze post injury. F) Heat map analysis on elevated plus maze indicating location and exploratory time within the arms of the elevated plus maze post injury. Graphs represent the A-C) mean ± SEM and D-E) median with interquartile range, (n= 12-14 per group; aa p<0.01 compared between injury groups).Heat maps are from group composites.

2.3. *Depressive-like behaviour*

Depressive-like behaviour was assessed through the forced swim test. Animals showed no significant main group effect in immobility time ($F_{3,50}$ = 1.434, p= 0.244) (Fig 3A), latency to first immobility ($F_{3,50}$ = 0.443, p= 0.723) (Fig 3B) or number of immobility episodes (H=2.104, p= 0.551) (Fig 3C) at 12 months post injury.

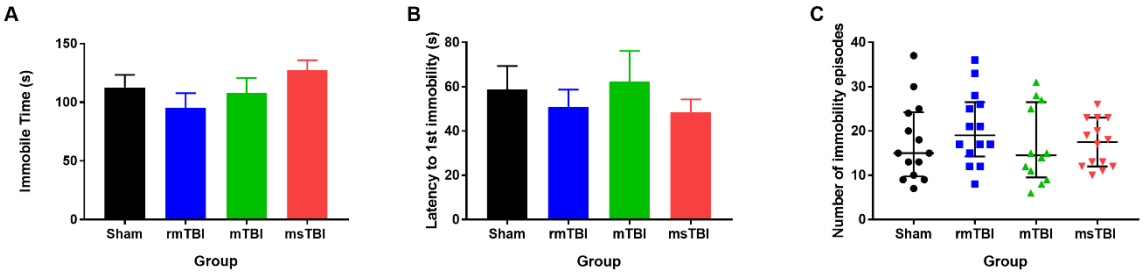

Figure 3: Depressive-like behaviour as measured in forced swim test by A) time spent immobile (s), B) latency to first immobility (s) and C) number of immobility episodes post injury. Graphs represent the A-B) mean ± SEM and C) median with interquartile range, (n= 12-14 per group).

2.4. *Cognition*

Cognitive outcome was assessed using the Y-Maze for spatial memory and Barnes maze for learning, memory (reference and working memory) and cognitive flexibility (ability to reprogram previously learned task) (Darcet et al., 2014). Our results showed no significant main effect in any of the Y-maze parameters between the TBI groups and shams at 12 months post injury; novel preference ($F_{3,50}$ = 1.234, p= 0.307) (Fig 4A), number of novel arm entries (H= 4.848, p= 0.183) (Fig 4B) and latency to 1st novel arm entry ($F_{3,48}$ = 0.0703, p= 0.976) (Fig 4C), as confirmed via heat-map analysis, with all animals showing greater intensity of staining in the novel arm, indicating higher occupancy of this arm (Fig 4D).

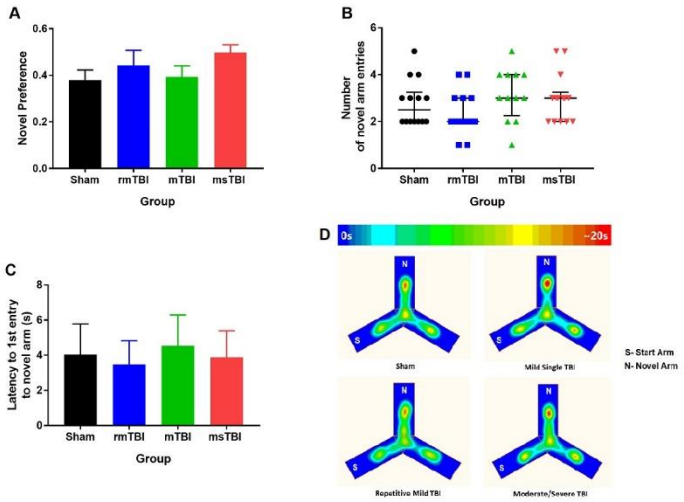

Figure 4: Cognition assessed through Y-maze for spatial working memory measured by A) novel preference, B) number of entries into the novel arm and C) latency to first novel arm entry post-injury. D)Heat map analysis on Y-maze indicating location and exploratory time within the arms of the Y-maze post injury. Graphs represent the A & C) mean ± SEM and B) median with interquartile range, (n= 12-14 per group). Heat maps are from group composites.

As for the Barnes maze, learning acquisition showed neither a significant group effect within the three days of trial acquisition ($F_{3,50}$ = 2.208, p= 0.099) nor a significant interaction effect ($F_{6,100}$ = 1.036, p=0.407), but, as would be expected, showed a significant main effect of time (trial days) ($F_{2,100}$ = 64.57, p<0.0001) (Fig 5A). Indeed, all groups showed a significant improvement in escape latency from day 1 to day 2 (p<0.05), with no significant differences noted between day 2 to 3 (Fig 5A). On the heat-map, this can be seen as the more targeted time spent near the escape box on Days 2 and 3, compared to the more exploratory pattern on Day 1 (Fig 6). There was also no significant main effect of group in latency to the old escape box location in trial 1 on probe day ($F_{3,38}$ = 0.346, p= 0.792) (Fig 5B). However, there was a significant group effect on cognitive flexibility, as indicated by time to find the new escape box, on probe day ($F_{3,38}$ = 4.343, p= 0.01). In trial 1 on probe day, the moderate/ severe TBI group had a significantly longer latency to reach the new escape box location when compared to shams (90.2 ±13.44 secs vs 48.08 ±6.96 secs, p= 0.009) (Fig 5C), which was also seen in trial 2 on probe day, but which did not reach statistical significance (57.5 ± 12.25 secs vs 28.5 ± 5.07

secs, p= 0.121). This is illustrated by the construction of a heat-map showing the average time spent in each part of the Barnes Maze across the two trials, with the moderate/severe TBI animals spending more time in the old escape box location (Fig 7). Similar to the acquisition trial, there was no significant interaction effect on the probe day ($F_{3,38}$ = 0.724, p= 0.544) but there was a significant main effect on time (difference between trials) ($F_{1,38}$ = 27.93, p<0.0001), with all animals improving their escape latency from trial 1 to trial 2 (p<0.05). Revisits to the old escape box location in trial 2 on probe day only showed a trend towards significance in effect between groups (H= 7.403, p =0.06) (Fig 5D). There was also no significant main effect seen between the groups in terms of reference memory error ($F_{3,38}$ = 0.932, p= 0.435) (Fig 5E) or working memory error ($F_{3,35}$ = 0.492, p= 0.69) (Fig 5F) on the probe day. Repetitive mild TBI and mild TBI groups showed no significant cognitive impairment when compared to shams on any of the cognition parameters (p > 0.05).

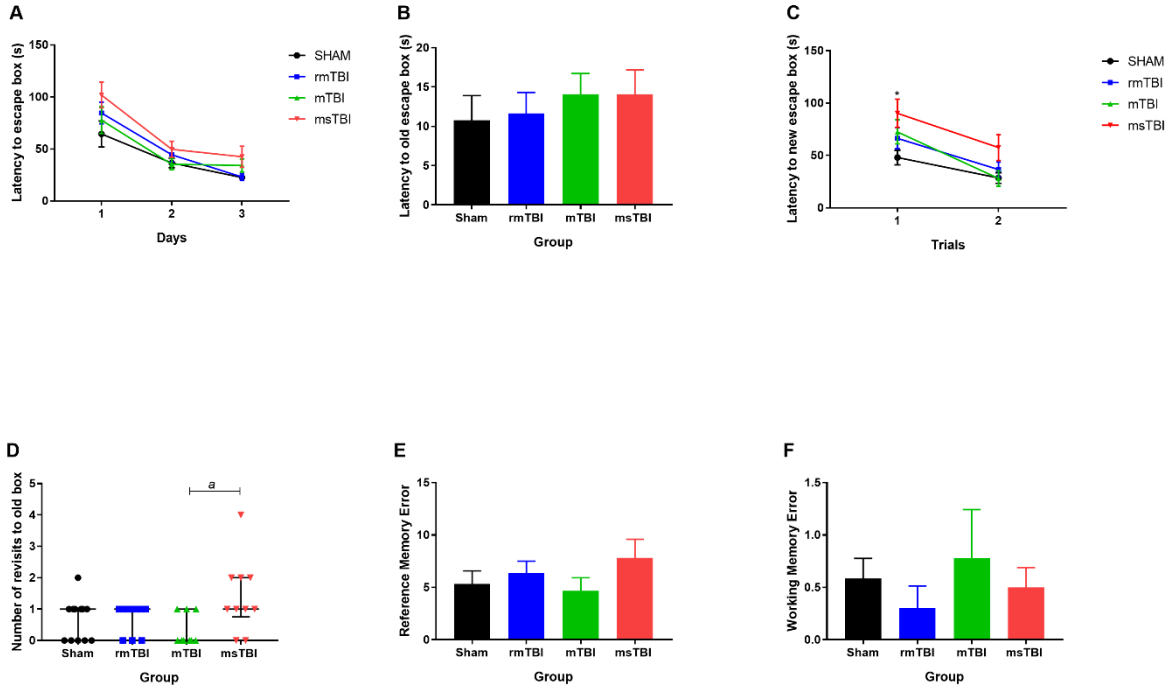

Figure 5: Cognition post-injury assessed through Barnes Maze for learning measured by A) latency to escape (s) on acquisition day, for memory measured by B) latency to old box location (s) on trial 1 probe day, D) number of revisits, E) reference memory error and F)working memory error on trial 2 on probe day, as well as for cognitive flexibility measured by C) latency to escape to the new box (s) on probe day. Graphs represent the A-C, E-F) mean ± SEM and D) median with interquartile range, (n= 12-14 per group).a p<0.05 compared between injury groups, *p<0.05 compared to shams).

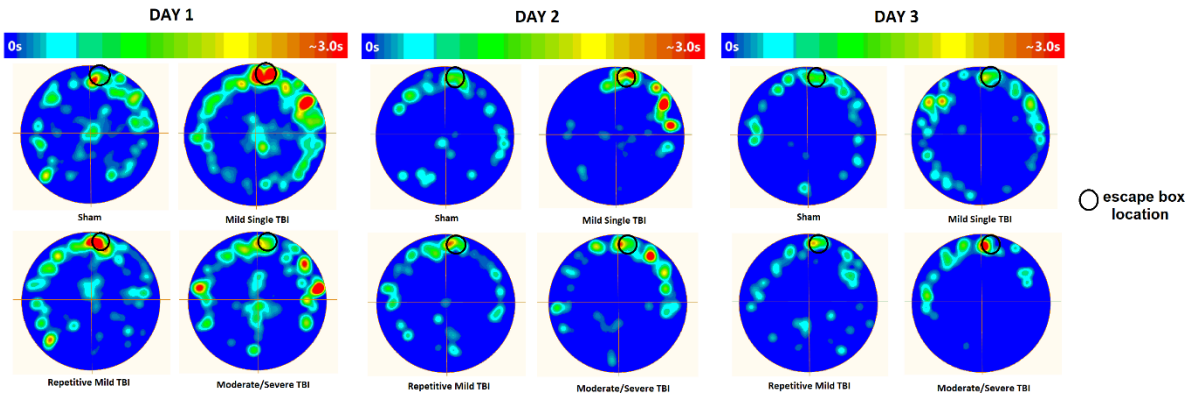

Figure 6: Heat map analysis on Barnes maze indicating location and exploratory time within the Barnes maze on acquisition days post injury. Heat maps are from group composites.

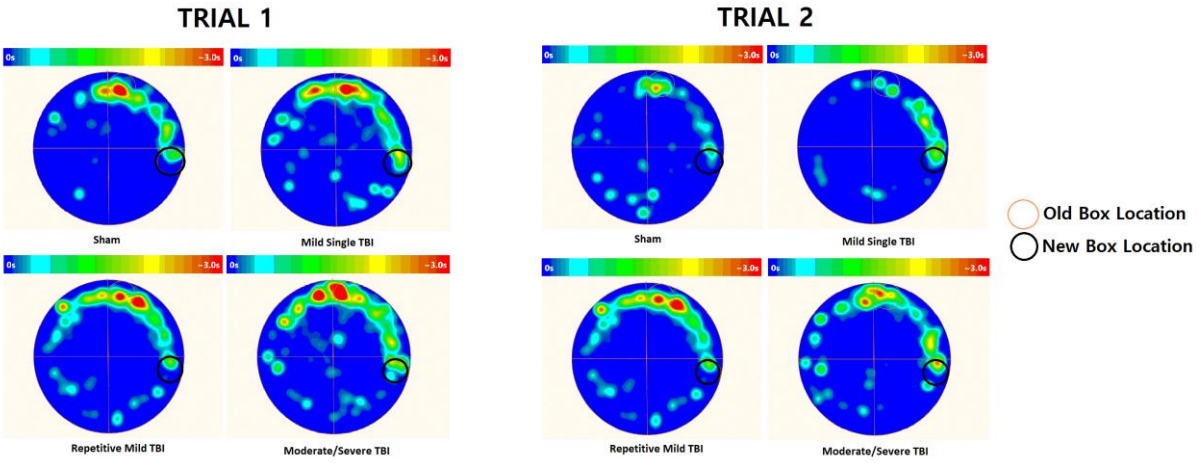

Figure 7: Heat map analysis on Barnes maze indicating location and exploratory time within the Barnes maze on probe day post injury. Heat maps are from group composites.

2.5. *Molecular Analysis*

NeuN was used to assess whether TBI led to loss of neurons at 12-months post-injury in the PFC. There were no significant changes in the total number of neurons observed in the PFC ($F_{3,21}$=2.329, p=0.104) (Fig 8A). This was further probed using several markers, including synaptophysin for assessing synaptic integrity, neurofilament light chain (NF-L) for assessing neurofilament structure and axonal stability and myelin basic protein (MBP) for assessing neuronal myelination stability. There were no alterations in synaptophysin levels in the PFC ($F_{3,21}$=0.244, p=0.865) (Fig 8B). Similarly, neither levels of NF-L ($F_{3,21}$=0.762, p=0.528) (Fig 8C) nor MBP ($F_{3,21}$=0.473, p=0.705) (Fig 8D) differed as a function of injury at 12 months post injury.

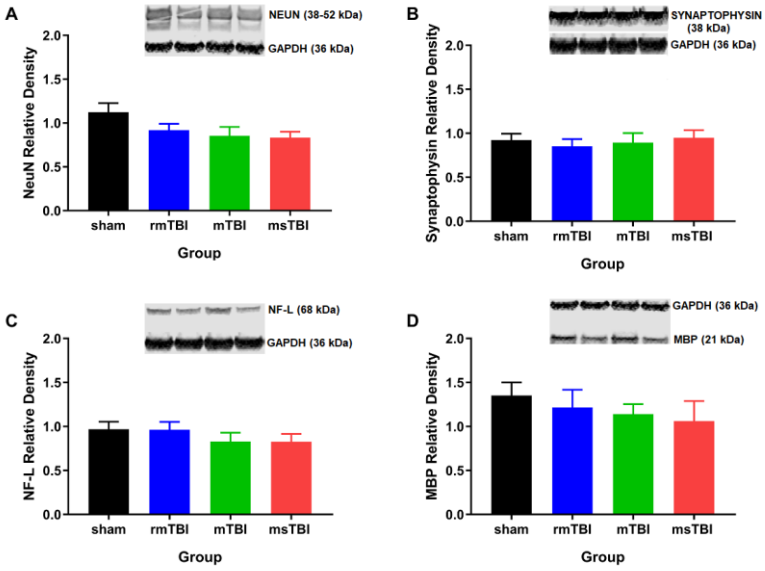

Figure 8: Molecular analysis on the prefrontal cortex at 12 months was measured using semi-quantitative western blotting to analyse A) neuronal survival (total neurons, NeuN marker), B) integrity (synaptophysin marker) and C-D) structure damage (NF-L and MBP markers). GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. Representative images of the western blots were extracted from Image Studio Lite.

3. Discussion

The current study investigated the presence of functional impairments at 12 months post-TBI of different severities; mild TBI, mild repetitive TBI and moderate/severe TBI. At 12 months post-DAI, when compared to age-matched sham animals, neither impairments in general locomotor activity, the expression of depressive-like behavior nor impairments in cognition in terms of spatial learning, working memory or recognition memory were evident, regardless of TBI severity. However, the moderate/ severe TBI animals exhibited significant subtle impairments in cognitive flexibility when compared to shams. There was also a trend towards reduced anxiety, as evidenced by more time spent in the open arm of the EPM, in the single mild TBI group, with significant differences between this group and both the repeated mild and single moderate-severe TBI animals, although no differences were seen in comparison

to sham-injured animals. This was further reflected in locomotor activity, with mild TBI animals having higher levels of activity compared to both repetitive mild TBI and moderate/severe TBI animals. Given the subtle alterations in cognitive flexibility at 12-months post-moderate/severe TBI, we used Western blot to analyse whether these were associated with changes in neuronal number or morphology in the PFC. Interestingly, no changes in either neuronal number or neuronal/synaptic integrity were found in the PFC at 12-months post-injury in any of the experimental groups. Taken together, the results of the current study seem to suggest that brain injury during early life has minimal effect on mid-life motor, cognitive or neuropsychiatric function, although subtle impairments in cognitive flexibility may still set the stage for the later emergence of more significant behavioural impairment.

The most notable finding in this study was that moderate-severe TBI led to a subtle impairment in cognitive flexibility at 12 months post-injury, with no effect seen on either spatial or recognition memory. This may indicate preferential disruption of prefrontal cortex function, as this region is critical for executive function, which governs cognitive flexibility (Bizon et al., 2012; Kim et al., 2011). Indeed, TBI has been consistently identified as a risk factor for higher-order cognitive deficits involving the frontal and prefrontal cortices (Nolan et al., 2018a). In healthy adults, tasks like the Trail Making Test-B, which require cognitive flexibility and switching attention, lead to activation of the dorsolateral prefrontal (DLPFC) and medial prefrontal regions of the brain (Zakzanis et al., 2005). Following TBI, performance on Trail Making Test-B, as well as other measures of cognitive flexibility, attention and working memory, such as the Hayling, Selective Attention Task, n-back and Symbol Digit Modalities Test, is slowed (Owens et al., 2018). This slowed information processing speed post-TBI is associated with lower fractional anisotropy (FA) and higher mean diffusivity (Bamdad et al.) scores, indicating white matter abnormality, in the majority of tracts assessed (Owens et al., 2018). Consistent with this, Ware and colleagues (2018) recently demonstrated that veterans who have suffered blast-induced TBI display elevated quantitative anisotropy (QA) and reduced right hemisphere volume in all subcortical-DLPFC tracts assessed, with decreased fibre

count in the right-DLPFC-putamen tract and increased generalized FA in the right DLPFC-thalamus tract specifically (Ware et al., 2018).

Similar effects on cognitive flexibility and prefrontal cortex function following a single moderate/severe TBI have also been reported preclinically (Robinson et al., 2018). In a model of lateral fluid percussion, working memory, as assessed by a T-maze task, was significantly impaired up to one week post-TBI, an effect that was accompanied by alterations in prefrontal cortex function (Smith et al., 2015a). Similarly, previous work from our group has shown impairments in cognitive flexibility at 3 months following a DAI (Arulsamy et al., 2018b). More chronically, in a CCI model that produced frontal contusions, impairments in reversal learning were observed at 12 months post-injury in a rule shift assay, a measure of cognitive flexibility (Chou et al., 2016b). It is not known, however, whether this deficit persisted from the time of injury or emerged at some later time-point prior to testing at 12 months, with further studies needed to incorporate a temporal time-course of behavioural changes required.

Despite the subtle alterations in cognitive flexibility observed in this study, there were no changes in the PFC in either total neuronal number, as measured by NeuN, or neuronal morphology, as measured by levels of synaptophysin, NF-L or MBP, at 12 months post-moderate/severe injury. This is consistent with earlier findings from our group, which showed no changes in neuronal morphology in the PFC at 3 months following moderate/severe TBI in the same experimental model of DAI (Arulsamy et al., 2018b). However, it is important to note that we conducted only a gross characterisation of neuronal morphology changes using WB analysis of total level of protein for each marker of interest. It is possible that a more in-depth analysis using IHC or neuroimaging techniques would have detected subtle changes in neuronal morphology or circuit connectivity, which are more likely to be present than gross alterations. For example, previous work in the lateral cortical impact model has demonstrated working memory dysfunction without the presence of neuronal cell death in the prelimbic region of the medial PFC [55], suggesting that more subtle alterations may drive these changes in PFC-

mediated cognition. In support of this, Hoskison *et al* found significant shortening of layer V/VI basal dendritic arbours and an increase in the density of both basal and apical dendritic spines in the prelimbic region of the medial PFC of rodents at 4 months following a lateral cortical impact injury (Hoskison et al., 2009). These subtle dendritic changes were accompanied by persistent alterations in working memory function on both delayed match-to-place and delayed alternation t-maze tasks (Hoskison et al., 2009). Thus, future studies should investigate subtle alterations in neuronal morphology within different cortical layers and specific subregions of the PFC, as well as the connectivity of the PFC with downstream structures.

Interestingly, in contrast to the findings of the current study, impairments in cognitive flexibility and alterations in PFC function have also previously been reported following rmTBI (Nolan et al., 2018a). In the CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) model, a newly developed model of injury allowing precise control of injury direction and impact velocity, mice showed impaired in several PFC dependent functions, including social memory (27-28 days post-injury) and impaired spatial working memory (32-35 days post-injury) following five repeated mild hits (Nolan et al., 2018a). These behavioural impairments were accompanied by a slight decrease in the adaptation rate of layer V pyramidal neurons in the mPFC (Nolan et al., 2018a). Similarly, work from our own group has demonstrated mild cognitive impairments up to 3 months post-injury in a model of rmTBI (McAteer et al., 2016). However, it is important to note that neither of these studies investigated the long-term time point (i.e. 12 months) being investigated in this study. It is possible that impairments in executive function following rmTBI may steadily improve over time, normalizing by 12 months, and may worsen again later with ageing. Consistent with this theory, in individuals who have suffered a moderate/severe TBI, measures of executive control function improve over time in the first year post-injury, only to decline again from this point (Vasquez et al., 2018).

In contrast to the subtle alterations in cognitive flexibility demonstrated in the current study, other types of cognitive function, such as novel arm recognition in the Y-maze and learning the escape location in the Barnes Maze, known to be dependent on hippocampal functioning, were intact at 12 months post-injury in this model of DAI. Previous studies have shown that damage to the hippocampus through administration of kainic acid impairs performance on the Y Maze, with no preference seen for the novel arm (Conrad et al., 1996), and that greater degrees of hippocampal loss following focal TBI are associated with worsening performance on the Barnes Maze (Corrigan et al., 2012). Thus, the lack of impairment in these tasks seen in the current study would suggest an intact hippocampus. Indeed, previous studies of diffuse moderate-severe TBI have shown a lack of hippocampal cell loss (Hallam et al., 2004) and preservation of hippocampal synaptic proteins (Arulsamy et al., 2018b), with a concomitant lack of hippocampal dependent cognitive deficits on either the MWM or radial arm maze following impact-acceleration TBI (Hallam et al., 2004; Maughan et al., 2000). This is in contrast, however, to focal injury models, with cognitive impairment on the Morris Water Maze seen at 12 months post-CCI (Dixon et al., 1999) and FPI (Pierce et al., 1998), in line with the significant hippocampal damage induced following these injury types (Broadbent et al., 2004; Conrad et al., 1996). More recently, these hippocampal-dependent cognitive deficits have been shown to persist in mice up to 6.5 months post-injury following 3 impacts over 3 days in the CHIMERA model (Chen et al., 2017b). Given that TBI is associated with the later development of dementia, it may be that the 12 month time-point is insufficient to detect hippocampal deficits in a DAI model. Indeed, given that the animals are 14-15 months old at the conclusion of this study, this represents only later middle-age in humans, with perhaps more time needed to develop hippocampal pathology sufficient to lead to detectable cognitive deficits. Thus, future studies are needed to investigate whether subtle changes in neuronal morphology or connectivity may be present in the hippocampus at this chronic timepoint, even in the absence of overt behavioral change.

In the current study, no alterations in depressive-like behaviour were noted following any injury type, despite evidence to suggest that both rmTBI and moderate/severe TBI can increase the risk for depression (Petraglia et al., 2014; Washington et al., 2012). Clinical studies show that there is a 40% prevalence of depression manifesting within a year post single moderate-severe TBI (Hudak et al., 2011; Jorge et al., 2004; Osborn et al., 2014a) and a three-fold increase risk of developing depression following repeated mild injuries (Guskiewicz et al., 2007). Pre-clinical studies have also reported increased immobility time in the forced swim test up to 3 months following both a single moderate diffuse TBI (Arulsamy et al., 2018b; Milman et al., 2005) and rmTBI (3 injuries in 10 days) (Corrigan et al., 2017b; McAteer et al., 2016), which suggests that this is a subacute deficit that has resolved by 12 months post-injury. Indeed, this is in line with other studies, with Jones *et al* (2008) reporting no behavioural despair (measured by FST) in their FPI animals at 6 months post injury (Jones et al., 2008). Alternatively, the FST may not be an appropriate test to investigate depressive-like behavior at this time point, as the large size of the animals impedes swimming behaviour. Use of other measurements, such as the saccharin preference test or other test of depressive-like behaviours in rodents, may be needed to confirm the lack of depressive-like phenotype at chronic time-points.

Our study also demonstrated a lack of anxiety-like effect on both the OFT and EPM in the single moderate severe TBI animals and repeated mild TBI animals. Intriguingly, there was a trend towards decreased anxiety in the single mild TBI group in open arm time in the EPM, with significant differences between this group and both the repeated mild and single moderate-severe groups. This increased time in the EPM may be seen as decreased anxiety (Walf and Frye), or may reflect disinhibition or increased impulsivity (Lindemann et al., 2007). Indeed, similar findings have previously been reported acutely following mTBI (Nolan et al., 2018a; Shultz et al., 2011), suggesting that this may be a particular behavioural consequence of this type of insult that can persist to the chronic phase. The interpretation of these results as disinhibition or increased impulsivity may be supported by the increased locomotor activity noted in the single mild TBI animals, as they had a significantly greater distance travelled than

the repetitive mild TBI animals in the OFT and had a significantly longer distance travelled than both of the other TBI groups in the EPM. This increased locomotor activity can be seen as hyperactivity (Budinich et al., 2013; Tucker et al., 2016; Yu et al., 2012), which is related to lesions in the cerebral cortex, mainly at the axis connecting the olfactory bulb and enthorhinal cortex within the pre-frontal cortex (Viggiano, 2008). This hyperactive behaviour (increased distance travelled) on the open field has been reported previously after mild CCI in mice (Budinich et al., 2013; Tucker et al., 2016), supporting our findings. It is unclear why this phenotype was present in the single mild TBI and not the other injury groups, with the possibility that the difference could also reflect slight locomotor deficits in the repetitive mild TBI and moderate/severe TBI group. Nevertheless, since this behaviour was only present in the single mild TBI group and was not significantly different than shams, one must be careful to not over-interpret this result and future studies will be necessary to further investigate specific neuropsychiatric impairments at chronic timepoints post-TBI.

In conclusion, our study shows that a moderate/ severe diffuse TBI may lead to significant impairments in cognitive flexibility at 12- months post-injury, suggestive of potential subtle alterations in either the structure or connectivity of the PFC that must be confirmed with future studies. In contrast, hippocampal dependent tasks that rely on spatial recognition memory were unaffected in all injured animals, indicating potential preservation of this region at 12 months post-injury. Surprisingly, no long-term meaningful behavioural effects of either single or repetitive mild injuries were noted at 12 months post-injury. It is important to note, however, that the current study used all male rodents. Given the growing body of literature indicating differences in injury outcomes in males versus females, additional work is needed to determine whether the behavioural changes seen chronically following TBI present differently as a function of sex. Furthermore, future studies should investigate the extent to which the ageing process itself contributes to the emergence of cognitive change following TBI.

Taken together, the results of this study provide the first systematic comparison of the functional effects following different severities of diffuse TBI in a preclinical model of DAI at one year post injury. While behavioural effects were subtle at this timepoint, indicating that DAI in early life has minimal effect on mid-life function, regardless of initial injury severity, differences observed between injury severity groups may still provide meaningful information. The risk for impaired cognitive function may be greater following moderate/severe TBI than more mild forms of injury, which may have important implications for risk of dementia development following different severities of injury. This is particularly important given that it is still impossible to predict which individuals will go on to develop dementia following TBI. Thus, understanding the temporal profile of even subtle alterations in behaviour following different severities of TBI may have clinical utility in helping to determine risk profiles.

4. Materials and Method

4.1. *Animals*

Male Sprague-Dawley rats (10-12 weeks) were used under the approval of the University of Adelaide Animal Ethics Committee (M-2015-243A) and (M-2015-187). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery (n=7), repetitive sham surgery (3 incisions at 5 day intervals) (n=7), a single mild diffuse TBI (n=12), repetitive mild diffuse TBI (3 mild diffuse injuries at 5 day intervals) (n=14), or moderate/severe diffuse TBI (n=14). Animals underwent a comprehensive functional battery assessing motor, neuropsychiatric and cognitive function at 12 months post injury.

4.2. *Injury Model*

The Marmarou impact-acceleration model (Marmarou et al., 1994) was utilized, as it has been extensively validated as a model of diffuse injury (Xiong et al., 2013b). Animal weights ranged from 420-480g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. Animals in the sham,

repetitive sham, mild diffuse TBI and repetitive mild diffuse TBI groups were maintained on 2% isoflurane via nose cone throughout, while animals in the moderate/severe diffuse TBI group were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout (Marmarou et al., 1994; McColl et al., 2018). A midline incision on the scalp was made to facilitate the placement of a metal disc centrally between lambda and bregma on the skull. Animals in the sham and repetitive sham groups received the incision only, with repetitive sham animals receiving the incision three times, with 5 day intervals between each incision.

Animals in the repetitive mild diffuse TBI and mild diffuse TBI group were removed from the nose cone and strapped onto a foam, with injury induced by releasing a 450g weight from a height of 0.75 metres down a clear tube onto the centre of the metal helmet; mild diffuse TBI animals receive this procedure only once, while repetitive mild diffuse TBI animals receive this injury three times, with 5 day intervals between each injury (Table 1). Conversely, animals in the moderate to severe diffuse TBI group were transiently taken off ventilation after incision, strapped onto a foam, with injury induced by releasing a 450g weight from a height of 2 metres (Table 1). Contact was observed to ensure a single, direct impact without a rebound hit in all animals. Only animals in the moderate/severe diffuse TBI group were then subjected to hypoxic conditions (2L/min nitrogen; 0.2L/min oxygen) for 10 minutes, to replicate the clinical effects seen following this injury model without ventilation, as this hypoxic condition is known to exacerbate the severity of the injury (Hellewell et al., 2010; Ishige et al., 1987a). Hypoxia alone had similar levels of cytoskeletal structure and neuroinflammation as shams under normoxic ventilation, as reported previously by Hellewell *et al.* (Hellewell et al., 2010). Saline treatment (5mL of 0.9% (w/v) saline solution) was administered subcutaneously to prevent dehydration (Eakin et al., 2015a) in the moderate/severe diffuse TBI group after wound closure, as well as if there was continuous weight loss post injury.

Table 1. Injury model and specifications

Injury Type	***Weight of metal***	***Height of drop***	***Days of injury***	***Mechanically ventilated***	***Hypoxia Treatment***	***Saline Treatment***
Repetitive Mild TBI	450g	0.75 m	3 days (at 5 day intervals)	No	No	No
Mild TBI	450g	0.75 m	1 day	No	No	No
Moderate to Severe TBI	450g	2.00 m	1 day	Yes	Yes	Yes

Wound closure was performed with surgical staples. Successful induction of moderate/severe TBI was assessed 24 hours later by rotarod scores of below 100, weight reduction of 5-10% and clinical signs (paresis and hunched posture). Animals in the moderate/severe TBI group that did not meet the above criteria were excluded from the study. Moderate/severe TBI was associated with a 20% mortality rate due to brainstem haemorrhage, which is similar to other weight-drop model studies of moderate to severe TBI (Hsieh et al., 2017). Shams, repetitive shams, mild diffuse TBI and repetitive mild diffuse TBI animals assessed at the same timepoint (24 hours) exhibited none of the clinical signs outlined above and had rotarod scores of more than 100s. Over the 12-month time period of the study, an additional 4 animals were lost due to age-related health complications.

4.3. *Functional studies*

Functional tests assessing cognition, anxiety, depression and motor function were performed at 12 months post-injury. All functional data was recorded using the ANY-maze Video Tracking System version 4.99m (Stoelting Co.). The functional tests were done in order from least to most aversive (stress inducing) to the animals. The experimenter was blinded to the experimental groups of each animal throughout the duration of the study, with unblinding only occurring during analysis of the results.

4.3.1 Open Field Test

The open field test (OFT) is a common test of locomotor activity (Malkesman et al., 2013). Animals were placed in the centre of a large square box (95cm x 95 cm) with walls at height 44.5cm and the total distance travelled over a 5 minute period was recorded. Time in centre of the field and rearing time were also measured for anxiety-like behaviour.

4.3.2 Elevated Plus Maze

The elevated plus maze (EPM) is widely used in anxiety research (Malkesman et al., 2013). Animals were placed in the centre of an elevated (50cm in height) cross-shaped maze consisting of two open and two closed maze arms (walls of height 40cm, each of length 50cm), facing the open arms, for 5 minutes. Time spent in the closed arms versus open arms as measured by the centre point of the animal's body was recorded, with increased time spent in the closed arms thought to represent anxiety-like behaviour (Malkesman et al., 2013). Other anxiety-like behaviour parameters measured in the EPM include number of centre crossings and number of open arm entries as measured by the centre point of the animal's body.

4.3.3 Y-Maze

The Y-Maze is used to test cognition in terms of spatial recognition memory (Wright and Conrad, 2005). In the Y-Maze, animals are placed in an equal angled Y-shaped arena, with each arm of the maze identical in size and shape, but visually distinct (due to cues on the wall), from the others. The test involves two 3-minute trials separated by 1 hour. In the first trial, one arm was closed off with a clear wall (novel arm) to enable the animal to visually recognise its location; in the second trial, this novel arm became accessible (wall removed). In cases of reduced spatial reference memory, the animal spends less time within the novel arm (Wolf et al., 2016).

4.3.4 Barnes Maze

The Barnes maze evaluates spatial learning and memory in rats (Sunyer et al., 2007). The maze is an elevated, open circular black platform of 1.2m in diameter with 18 holes evenly distributed

along its edges. One of the holes is pre-determined as the escape hole, with a black escape box placed below the hole. The Barnes maze test was preformed over the course of five days; three days of acquisition trials, a rest day (no interaction with the animals) and a probe day. During the acquisition days, animals were subject to two trials spaced 15 mins apart. They were placed in the centre of the Barnes maze in a brightly lit room, with the time taken for the animal to find and enter the escape box recorded. On day 5, the escape box was relocated to a new hole and two trials were conducted 1 hr apart. In trial 1, the time taken for the animal to reach the old position of the escape hole was recorded. In both trials, the time taken to locate and enter the newly relocated escape box was recorded as a measure of cognitive flexibility. Number of revisits to the old box location on trial 2 of probe day, working memory error (measured as the number of revisits to the same hole after exploration of less than 3 different holes) and reference memory error (measured as the number of visits to any of the holes that was not the escape hole) were recorded as additional cognitive parameters.

4.3.5 Forced Swim Test

The forced swim test (FST) is widely utilised to assess depressive-like behaviour (Bogdanova et al., 2013). The animal was placed within an inescapable glass cylinder filled halfway with 25 °C water, adjusted for the animal's length so that the hind legs do not touch the bottom of the cylinder, for 5 minutes. The time spent immobile, number of immobile episodes and latency to first immobile episode were recorded as a measure of behavioural despair.

4.4 *Tissue Collection and Processing*

Animals were transcardially perfused with 0.9% saline and the brain was removed. The prefrontal cortex (n=5-7 per group) was dissected and snap-frozen in liquid nitrogen before being stored at -80°C.

The samples were taken out and homogenised in freshly prepared RIPA lysis buffer (150mM sodium chloride, 50mM Tris-hydrochloride acid of pH 7.5-8, 1% of NP-40 IGEPAL CA-630,

0.5% sodium deoxycholate, 0.1% of sodium dodecyl sulfate (SDS) and distilled water) with 1X cOmplete™ EDTA-free protease inhibitor cocktail (Sigma). After homogenisation, each sample underwent 3 bursts of 10 seconds duration under a sonicator probe with a cooling period between each burst. Then the samples were centrifuged for 30 minutes at 14000 rpm and 4°C, before the supernatant were collected. Protein concentration was estimated with Pierce BCA Protein Assay Kit (ThermoScientific) with the absorbance read at 540nm. All supernatant were stored at -80°C until further usage.

4.5 *Western Blot*

Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels (Life Technologies) with 30µg of protein loaded per well. Gels were run at 150V for 1 hour. After the run, blots were transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes x 5 minutes), stained with Ponceau S red solution (Fluka Analytical) (5 minutes) for protein visualisation, and washed with distilled water until sufficient removal of the Ponceau stain had been achieved.

Membranes were then incubated for 5 minutes with the 1X iBind solution before proceeding with the final step of simultaneous incubation with primary and secondary antibodies in 1X iBind solution for 2.5 hours using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations; synaptophysin (1:4000, Abcam, ab32127), neurofilament light-chain (1:2000, Abcam, ab72997), myelin basic protein (1:750, Abcam, ab62631) and NeuN (1:750, Abcam, ab177487) with housekeeper antibody GAPDH (1:1000, Abcam, ab83957 and 1:1000, Abcam, ab9485). Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. The blots were imaged using an Odyssey CLx Infrared Imaging System (model 9140) (LI-COR, Inc.) set at auto resolution for optimum visualisation. Semi-quantitative analysis of band signals were performed using Image Studio

Lite version 5.2. Normalization of blot runs were performed using a single control sample (Rapoport et al.) across blots of the same protein of interest. Thus, relative density of the samples was calculated based on the adjusted density for each blot, as below:

$$\text{Adjusted density} = \frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$$

$$\text{Relative density} = \frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$$

4.6. *Statistics*

All data, with the exception of Barnes maze data, was analysed via one-way ANOVA (Analysis of Variance) with injury severity as the between subjects factor using IBM SPSS statistics 24 and GraphPad Prism software. A repeated two-way analysis of variance was performed on the acquisition days and latency to new escape box on probe day of the Barnes maze test, with trial as the within subjects factor and injury severity as the between subjects factor. Post hoc testing was conducted using Tukey's method. The Kruskal Wallis test was used for non-parametric measurements. For all tests, p values < 0.05 were considered statistically significant. Shams and repetitive shams were combined together as a single sham group, as there were no statistically significant differences in any parameters of behavioural and molecular data.

Chapter 4
Age, but not severity of injury, mediates decline in executive function: Validation of the rodent touchscreen paradigm for preclinical models of traumatic brain injury

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Alina Arulsamy		
Contribution to the Paper	Performed analysis of behavioural data, interpreted data and wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/03/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Age, but not severity of injury, mediates decline in executive function: Validation of the rodent touchscreen paradigm for preclinical models of traumatic brain injury.

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AA was involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

Abstract:

Increasingly, traumatic brain injury (TBI) is recognised as not just an acute event but instead results in ongoing neuronal injury that may lead to chronic impairments in multiple cognitive domains. Of these, executive dysfunctions are one of the more common changes reported following TBI. To fully understand the relationship between TBI and executive dysfunction, experimental models are needed. However, to date, there have been a lack of preclinical studies systematically comparing the effect of injury severity on executive function, particularly at long-term timepoints. Furthermore, many previous studies have not used behavioural measures that are sensitive to the full range of executive dysfunction that may manifest after injury, particularly in models of diffuse axonal injury. The current study aimed to investigate the temporal profile, up to 12 months post-injury, of the evolution of executive dysfunction following different severities of injury in a diffuse axonal injury (DAI) model. We utilised a rodent touchscreen paradigm to administer the 5 Choice- Continuous Performance Task (5C-CPT), an extension of the 5-choice serial reaction time task (5CSRT). Interestingly, there were no differences in learning, motivation, attention, response time or impulsivity at 1, 6 or 12 months post-injury in any of the TBI groups compared to sham. Instead, most of the effects on executive function seen at the 12 month timepoint appeared to be a result of ageing, not injury. As even the 12-month timepoint represents middle age in the rat, future studies will be needed to determine whether DAI may influence the presentation of executive dysfunction in older age.

Keywords: cognitive flexibility, diffuse injury, learning, attention, motivation, reaction time

1. Introduction:

Traumatic brain injury represents one of the leading causes of death and disability worldwide. Approximately 2.5 million TBI-related emergency department visits, hospitalisations and deaths were reported by the Centre of Disease Control and Prevention in just 2010 alone (Faul et al., 2010; Frieden et al., 2014). Annually, 54-60 million people worldwide suffer from TBI either through falls, motor vehicle accidents or sports related concussion, with mild TBI accounting for at least 70% of TBI (Khan et al., 2003). Moreover, increasing evidence suggests that TBI is not just an acute event but instead results in ongoing neuronal injury (Carron et al., 2016; Gao and Chen, 2011; Sato et al., 2001) that may lead to chronic impairments in cognitive function (McKee et al., 2009; Mouzon et al., 2018; Till et al., 2008). In a Norwegian multicentre cohort study, at one year post-injury, cognitive impairment was still present in 67% of individuals who had experienced a severe TBI (Sigurdardottir et al., 2015). These impairments can manifest in multiple cognitive domains, including slowed information processing and impairments in attention, working memory, social cognition, long-term memory, self-awareness and executive function (Azouvi et al., 2017).

Of these, worsened executive function is one of the more common cognitive impairments reported following TBI (Bamdad et al., 2003). Executive function is comprised of a variety of components, including realistic goal-setting, goal-directed behavioural planning, cognitive flexibility, attentional switching, problem solving, behavioural inhibition, self-initiation, self-monitoring, self-awareness and strategic behaviour (Ylvisaker, 1998). Such functions are largely mediated by the pre-frontal cortex (PFC), an area of the brain particularly vulnerable to injury in TBI (McAllister, 2008). Using the Behaviour Rating Inventory of Executive Function (BRIEF), post-TBI impairments were noted by knowledgeable informants in five subtypes of executive function, with elevations on the Shift, Plan/Organise, Task Monitor, Organisation of Materials and, most severely, Working Memory clinical scales (Matheson, 2010). Similarly, neuropsychological studies have noted impairments in performance on multiple measures of cognitive flexibility, attention and working memory,

including the Trail Making Test-B, Hayling, Selective Attention Task, n-back and Symbol Digit Modalities Test, following TBI (Draper and Ponsford, 2008; Lange et al., 2005; Owens et al., 2016; Pearce et al., 2016). Such impairments are a major predictor of well-being, social function and quality of life in individuals with a history of TBI (Muscara et al., 2008b; Ubukata et al., 2017) as they make it particularly difficult for individuals to adapt their behaviour to changing situations and circumstances. In support of this, executive dysfunction was associated with lower community integration following injury in individuals with a history of TBI (Reid-Arndt et al., 2007). Executive dysfunction has also been shown to be a major predictor of employability post-injury (Weber et al., 2018).

Interestingly, executive function deficits may display a "dose response"- like pattern, with more severe injury associated with a greater risk of impairment (Fujiwara et al., 2008). In support of this, following moderate-severe injury, persistent cognitive deficits are noted in ~65% of patients (1999), while just 15% of patients report persistent impairment following mild TBI (Bigler et al., 2013), although a history of repeated concussive impacts has been linked to increased risk of executive dysfunction in later life (Montenigro et al., 2017). Within a group of blast-related mild TBI, severity of injury was also a significant predictor of dysfunction, with more severe injury associated with worsened integration of regions important for visual sensory input with frontal cortical regions important for executive function (Gilmore et al., 2016). However, not all literature has been consistent. In a study comparing decision making ability, as measured by performance on the Iowa Gambling Task, while differences were observed between individuals with TBI and control participants, these were not a function of injury severity (that is, performance did not differ between individuals with mild and severe TBI) (Cotrena et al., 2014). Similarly, despite the strong association between measures of executive function and life satisfaction, injury severity does not significantly correlate with quality of life assessment (Dijkers, 2004; Johnston and Miklos, 2002)

In order to fully understand the relationship between injury severity and the evolution of executive dysfunction over time post-TBI, including brain mechanisms that may account for

this, experimental models of injury are clearly needed. However, to date, there have been a lack of preclinical studies systematically comparing the effect of injury severity on executive function, particularly at long-term timepoints post-injury. Recently, our group reported persistent deficits in cognitive flexibility at both 3 months (Arulsamy et al., 2018b) and 12 months (Arulsamy et al., 2019) post-TBI in a model of moderate-severe diffuse axonal injury (Lv et al., 2014). Interestingly, while mild deficits in cognitive flexibility have previously been noted at 3 months following repetitive mild TBI (rmTBI; (McAteer et al., 2016), these deficits were not present at 12-months post-injury following either mild DAI or rmTBI (Arulsamy et al., 2019), further supporting the idea of a differential risk profile for impaired executive function based on the severity of the original insult. However, it is important to note that, even following moderate-severe injury, the deficits in cognitive flexibility observed at 12-months post-injury were fairly mild. Thus, it is possible that more subtle alterations in executive function may not have been picked up by the Barnes maze task utilised in this study and that more sensitive measures of executive function may be needed.

Despite the prevalence of executive dysfunction deficits in TBI, however, there have been relatively few investigations utilising sensitive measures of executive function in experimental models of TBI, as many studies to date have instead focused on characterising deficits in spatial learning (Bondi et al., 2014; Ozga et al., 2018). Previous studies in the controlled cortical impact (CCI) model have used a novel complex cognitive behavioural task, the attentional set-shifting task, analogous to the Wisconsin Card Sorting Test, to detect deficits in executive function and behavioural flexibility that increased as a function of injury severity at 4 weeks post-TBI (Bondi et al., 2014; Njoku et al., 2019). Similarly, in a CCI model that produced moderate-severe frontal contusions, a rule shift assay task has been used to demonstrate persistent deficits in cognitive flexibility, as measured by impairments in reversal learning, up to 5.5 months following injury (Chou et al., 2016a). Also in the CCI model, the Rodent Gambling Task, an analogue of the Iowa Gambling task, identified reductions in optimal decision-making, with a bias towards both riskier and safer, sub-optimal choices,

chronically (up to 12 weeks) following injury (Shaver et al., 2019a). Interestingly, chronic increases in impulsive decision making, as measured by performance on the Delay Discounting Task, did not vary as a function of the initial CCI injury severity (Vonder Haar et al., 2017). This is consistent with previous work in the 5-choice serial reaction time task (5CSRT) reporting persistent (up to 14 weeks post-injury) deficits in impulse control following even mild CCI, although it is worth noting that, while only this domain was impaired following mild injury, all cognitive domains were impaired following moderate- or severe-CCI injury (Vonder Haar et al., 2016). To date, however, sensitive measures of executive function have not been used to assess the behavioural effects of a purely diffuse injury. This is particularly significant, given that "pure" forms of focal injury occur in only 28% of moderate-severe TBI cases, while diffuse axonal injury is seen in 72% of individuals, with "pure" diffuse axonal associated with significantly lower scores on the Glasgow Coma Scale (Skandsen et al., 2010).

Thus, the aim of the current study was to investigate the temporal profile, up to 12 months post-injury, of the evolution of executive dysfunction following different severities of injury in an experimental model of DAI. In order to do so, we utilised a rodent touchscreen paradigm to administer the 5 Choice- Continuous Performance Task (5C-CPT), an extension of the 5CSRT. A number of executive functions can be assessed using this task, including attention (response accuracy), inhibitory control (premature responses) and processing speed (response and reward collection latency) (Carli et al., 1983) and inhibitory control (Young et al., 2009). Although these touchscreen tasks paradigms have been utilised to investigate cognitive dysfunction in other disease models, such as AD (Bharmal et al., 2015; Romberg et al., 2011; Romberg et al., 2013a; Young et al., 2009), to our knowledge, this study is the first to use the 5CSRT/5C-CPT touchscreen paradigm to investigate alterations in executive function in an experimental model of TBI. Given the prevalence, persistence and significance of executive dysfunction following TBI, developing sensitive tasks to better investigate the evolution of this impairment following different severities of injury is critically important.

2. Materials and Method:

2.1. *Animals*

Adult male Sprague-Dawley rats (10-12 weeks) were used under approval of the University of Adelaide Animal Ethics Committee (M-2015-187 and M-2015-243A). Animals were housed (2-3 animals per cage) under conventional laboratory conditions, with a 12-hour light-dark cycle (lights on at 7am and off at 7pm) and had access to water ad libitum. All experiments were performed in the light cycle. Food was restricted at 85-90% of free-feeding body weight throughout the experiment. Animals were randomly allocated to either sham or repetitive sham (rshams) surgery or to one of the diffuse TBI groups: mild single (mTBI), mild repetitive (3 mild single TBIs, with 5 days between injuries, rmTBI) or moderate-severe single (msTBI). Animals were then allocated to one of three timepoints for post-injury follow-up: 1 month post-injury (shams/rshams n=6 shams+6 rshams; mTBI n=10; rmTBI n=10; msTBI n=12), 6 months post-injury (shams/rshams n=7 shams+7 rshams; mTBI n=14; rmTBI n=13; msTBI n=14) or 12 months post injury (shams/rshams n=7 shams+7 rshams; mTBI n=12; rmTBI n=14; msTBI n=14).

2.2. *Injury Model*

The Marmarou impact-acceleration model (Marmarou et al., 1994) was utilized, as it has been extensively validated as a model of diffuse injury (Xiong et al., 2013a) (Figure 1). Animal weights ranged from 420-480g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. Animals in the sham, repetitive sham, mTBI and rmTBI groups were maintained on 2% isoflurane via nose cone throughout (Collins-Praino et al., 2018; Corrigan et al., 2017a; McAteer et al., 2016), while animals in the moderate/severe diffuse TBI group were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout (Arulsamy et al., 2018b; Arulsamy et al., 2019; Plummer et al., 2018). Once the animal was unresponsive to paw pinch, a midline incision was made to facilitate the placement of a metal disc centrally between lambda and bregma. Animals in the sham and repetitive sham groups received the incision only, with

repetitive sham animals receiving the incision three times, with 5 day intervals between each incision.

Animals assigned to undergo TBI were then transiently taken off ventilation (msTBI group) or removed from the nose cone (mTBI and rmTBI) and strapped onto a foam, with injury induced by releasing a 450g weight from a height of either 0.75m (mTBI and rmTBI) or 2m (msTBI) down a clear tube onto the centre of the metal helmet. Contact was observed to ensure single, direct impact without a rebound hit. While animals in the mTBI and msTBI groups received this procedure only once, animals in the rmTBI group received the injury three times, with 5 day intervals between each injury (Table 1). In addition to the injury procedure, animals receiving msTBI were also subjected to hypoxic conditions (2L/min nitrogen; 0.2L/min oxygen) for 10 minutes, in order to more closely model the clinical effects seen following this type of injury, as hypoxic conditions have been shown to worsen injury severity (Hellewell et al., 2010; Ishige et al., 1987b). Hypoxia itself is unlikely to be responsible for any differences seen between the TBI groups, as animals undergoing hypoxia alone have been shown to display similar cytoskeletal structure and levels of neuroinflammation to shams under normoxic ventilation (Hellewell et al., 2010). All other groups (sham, mTBI and rmTBI) received normoxic ventilation after injury.

Wound closure was performed with surgical staples. Saline treatment (5mL of 0.9% (w/v) saline solution) was administered subcutaneously to prevent dehydration in the msTBI group after wound closure, as well as if there was continuous weight loss post injury (Eakin et al., 2015b). Successful induction of moderate-severe TBI was assessed 24 hours post-injury by rotarod scores of below 100, weight reduction of 5-10% and clinical signs (paresis and hunched posture). Animals in the msTBI group that did not meet the above criteria were excluded from the study. Moderate/severe TBI was associated with a 20% mortality rate due to brainstem haemorrhage, which is similar to other weight-drop model studies of moderate to severe TBI (Hsieh et al., 2017). Shams, repetitive shams, mTBI and rmTBI animals assessed at the same timepoint (24 hours) exhibited none of the clinical signs and had rotarod scores of more than

100s. Over the 12-month time period of the study, an additional 4 animals were lost due to age-related health complications.

Table 1. Injury model and specifications

Injury Type	***Weight of metal***	***Height of drop***	***Days of injury***	***Mechanically ventilated***	***Hypoxia Treatment***	***Saline Treatment***
Repetitive Mild TBI	450g	0.75 m	3 days (at 5 day intervals)	No	No	No
Mild TBI	450g	0.75 m	1 day	No	No	No
Moderate to Severe TBI	450g	2.00 m	1 day	Yes	Yes	Yes

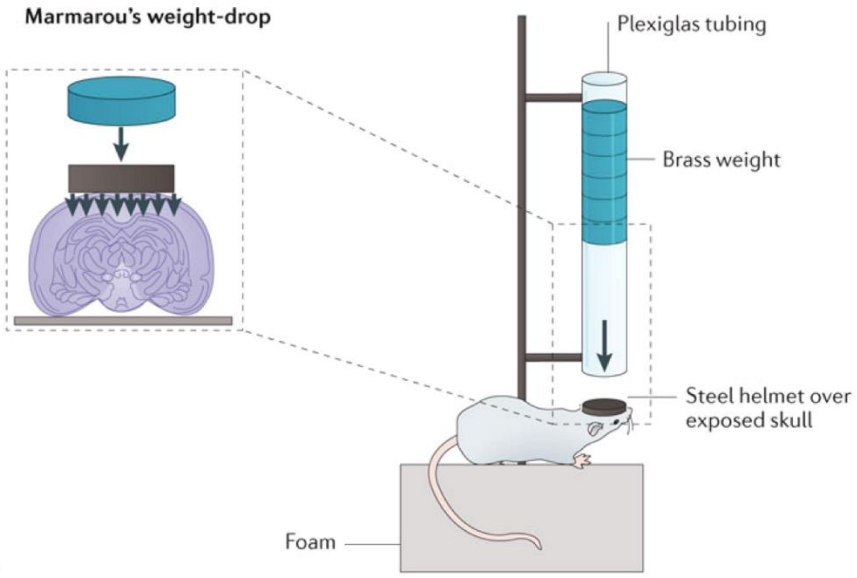

Figure 1: Marmarou's impact acceleration model of diffuse axonal injury. (Image adapted from Xiong *et al*, 2013)

2.3. *5-Choice Serial Reaction Time Task (5CSRT)/5 Choice- Continuous Performance Task (5C-CPT)*

Once achieving a reduction to 85-90% of free-feeding weight, animals were habituated, pre-trained, trained and finally probe tested on the touchscreen cognitive chamber apparatus at their respective follow-up timepoint (i.e. either 1, 6 or 12 months). Animals were only subjected to a gradual food restriction two weeks prior to their pre-training and were maintained at 85-90% of free feeding weight throughout the testing period. Pre-training and training sessions were

performed according to a set number of days (total of 25 days), in order to avoid overtraining in some groups/ subsets of animals. Probe testing was done on the 26th day. All stages of the experiment were performed between 9am and 2pm daily, with supplementary food given as needed one hour after completion of the experiment. All chambers were thoroughly cleaned with 75% alcohol after each use. Each animal was placed in the same assigned chamber throughout the experiment to avoid re-habituation.

2.3.1. Testing Apparatus

Behavioural testing was conducted in the Bussey-Saksida Touchscreen operant chamber (Campden Instruments Ltd.,U.K.) based on methodology described in previous reports using this task (Horner et al., 2013; Kim et al., 2015; Mar et al., 2013b; Young et al., 2009). The model (80604-20) consist of 4 identical chambers fixed in a (2 x 2) grid. Each chamber consists of a fan (for ventilation and prevention of noises from outside the chamber), a touchscreen monitor (15inch, screen resolution of 1024 x 768) on one side, a light equipped magazine unit and a pellet dispenser on the opposite side, a tone generator and a houselight (Brettschneider et al.) on the ceiling. The chambers are composed of three black plastic walls arranged in a trapezoidal shape (30cm height x 33cm length x 13cm width at magazine end x 25cm width at monitor end), with a perforated stainless steel floor raised above a collection tray. The shape of the chamber allows the animal to focus its attention on the touchscreen monitor for the stimulus light. A black mask with five response windows (3cm x 3cm) equally spaced at 1.5cm apart and positioned centrally at 2cm from the floor was fitted in front of the touchscreen to allow fixated response from the animal when a white solid square (of the same dimensions as the window) illuminates (known as the stimulus light). The apparatus was connected to a computer which uses the software “Whisker Server” to control and operate all the chambers simultaneously. ABETII program software that is equipped with pre-set task programs was used to run and measure the 5CSRT/5C-CPT task in the chambers.

2.3.2. Pre-training

Once acclimatized to the experiment room, the animals were weighed for three consecutive days with *ad libitum* food and water, in order to determine free-feeding weight. Food restriction according to animal ethics guidelines was performed, where the animal had to reach a goal weight of 85-90% of free-feeding body weight (~4g of food per 100g of body weight daily). Once goal weight was reached, sugar pellets (Dustless Precision Sugar Pellets, ASF0042, 45mg, Able Scientific, Australia), which serve as reward for the 5CSRT/5C-CPT task, were introduced in the home cage of the animal, as a means of habituating them to the pellets. The next day (Day 1), 10 pellets were placed in the magazine of each chamber, and the animals were allowed to habituate to their respective chamber for 30 minutes, with the houselight and stimuli kept on throughout. After 30 minutes, the animal was placed back into its respective home cage and the magazine was checked for complete consumption of food pellets. If complete consumption was achieved, the animal was allowed to move on to the next task. Otherwise, habituation was repeated.

After habituation was achieved, the animals were subjected to the pre-training tasks; initial touch and must touch, according to the settings outlined in Table 2. These settings were input into the 5CSRT task on the ABETII software and the task was run once the animals were all placed into their respective chambers. Number of food pellets, stimulus presentation, houselight, tone generation and outcome measurements were automatically controlled and recorded by the ABETII software. The criterion to pass the initial touch task on day 2 was finishing 30 trials in 30 minutes; then, on day 3, the must touch task was run. The criterion to pass the must touch task was to finish 20 trials in 30 minutes for two consecutive days, but due to the difficulty of the task for animals following TBI, animals took an average of 3-5 days to achieve this criterion. Once achieved, the animal was moved onto the training phase.

Table 2. Pre-training session variables (adapted from Rat Touch 5C-CPT ABETII Manual V1.2)

Session	Initial Touch	Must Touch
Session length (min)	30	30
Trials	30	20
ITI (Cotrena et al.)	0	5
Time out (Cotrena et al.)	0	0
Stimulus duration (Cotrena et al.)	30	-
Limited hold	-	-
Day	2	~3-7

ITI: inter-trial interval

2.3.3. Training

The training period was divided into two sections; the 5CSRT training and the 5C-CPT training (Table 3). The first section was the 5CSRT training period, which consists of the stimulus light being presented pseudo-randomly in any one of the windows for a decreasing duration of time (60, 30, 20, 10, 5, 2.5 secs) from session 1 to session 6, respectively (Table 3), in order to facilitate a good learning curve. On average, animals spent 20 days in total to go through the 5CSRT training period successfully, with animals that achieved success earlier (~15 days) allowed to rest until the 5C-CPT training phase. Only one session was run per day, in order to avoid overtraining. Criteria to pass each session was achieving at least 80% in accuracy and less than 20% in omissions.

Table 3. Training and probe session variables (adapted from Rat Touch 5C-CPT ABETII Manual V1.2)

Session	1	2	3	4	5	6	5C-CPT	Probe
Session length (min)	30	30	30	30	30	30	30	30
Trials	60	60	60	60	60	60	100	120 (80 go trials + 40 no-go trials)
ITI (Cotrena et al.)	5	5	5	5	5	5	5	5
Time out (Cotrena et al.)	5	5	5	5	5	5	5	5
Stimulus duration (Cotrena et al.)	60	30	20	10	5	2.5	2.5	0.5, 1, 2, 3, 4 secs randomly presented, equally
Limited hold	60	30	20	10	5	5	Go trial (5 sec) No-Go trial (3 sec)	Go trial (5 sec) No-Go trial (3 sec)
Day	~8	~9	~10	~11-12	~13-16	~17-20	21-25	26

ITI: inter-trial interval, 5C-CPT: 5 Choice-Continuous Performance Task

Each session began with a sugar pellet being released and the magazine light being switched on. When the animal retrieved the pellet, the magazine light was switched off. This was then followed by a 5 sec inter-trial interval and subsequent presentation of the stimulus light (with duration dependent on the session number; 60, 30, 20, 10, 5, 2.5 secs for sessions 1-6, respectively) in any one of the 5 windows. If the animal made a response during the 5 sec ITI (**premature response**), the houselights remained on, but no pellet was rewarded. Following the ITI, the animal either had to nose poke the right window when the stimulus was presented or within the specified limited hold time after the stimulus light had disappeared (Table 3). If the right window was nose poked (**correct response**), a tone was generated, a sugar pellet was released and the magazine light turned on. Conversely, if the wrong window was nose poked

(**incorrect response**), the houselights turned off for 5 secs (*timeout*) and no pellet was rewarded. If the animal did not make a response within the limited hold time (**omission**), there was a 5 sec timeout period, with the houselights turned off and no food pellet rewarded. Following a 5 sec ITI, the next trial would begin. Each training session was completed at the end of 60 trials or at 30 minutes, whichever came first.

After 20 days, animals that did not achieve a successful training phase (that is, where they did not pass the criteria for session 5 with a 5 sec stimulus duration) were excluded both from the 5C-CPT training phase and the probe test. In this study, 1 animal in the 1-month follow-up group (1 rmTBI), 6 animals in the 6-month follow-up group (1 sham, 2 mTBI and 3 msTBI) and 10 animals in the 12-month follow-up (3 sham, 4 rmTBI and 3 msTBI) failed to pass the criteria for session 5 and were excluded from 5C-CPT training, as well as from the overall analysis of this study.

The second section of the training phase, for the 5C-CPT task, began on the 21st day and was conducted for 5 consecutive days (day 21 to day 25). During this section of the training, the stimulus presentation duration was kept constant (2.5 sec) (Table 3). The session began similarly to the previous 5CSRT training, but consisted of two types of trials; Go trials and No-Go trials. The presentation of the stimulus light in all five windows is known as a *No-Go trial*, while the presentation of the stimulus light in only one window at a time is known as a *Go-trial* (Figure 2). When a No-Go trial was presented, the animal had to refrain from responding to any of the lighted windows during the 3 sec limited hold time (**correct rejection**). If the animal refrained from responding when all 5 windows were lit up for the entire 3 sec, then a tone was generated, the sugar pellet was rewarded and the magazine light switched on. Conversely, if the animal failed to inhibit its response (**false alarm**), the houselight switched off for a timeout period of 5 sec and no pellet was released. No-Go trials were pseudo-randomly interspersed with Go-trials, accounting for 1/3 of the total number of trials. The total number of trials for this section of the training phase was 100 trials in 30 minutes: 30 No-Go trials and 70 Go trials.

There were no achievement criteria for this section of the training phase, with all animals moving onto the probe test on the 26th day.

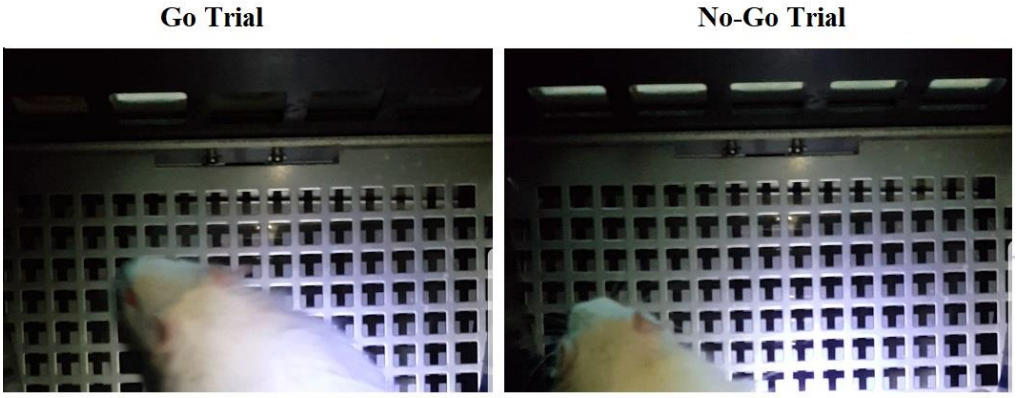

Figure 2: Touchscreen cognitive chamber utilizing the 5-Choice Continuous Performance task (5C-CPT). Go trials; light stimulus presented in one of the five windows and animal has to nose-poke the presented window, No-Go trials; all five windows are presented with light stimulus and animal has to inhibit from nose-poke response.

2.3.4. Probe test

On day 26th of behaviour testing, the 5C-CPT probe test was performed. The probe test consists of 120 trials, 80 Go trials and 40 No-Go trials, over 30 minutes. The stimulus duration varied between each trial pseudo-randomly (0.5sec, 1 sec, 2 sec, 3 sec or 4 sec). All settings, except the stimulus duration and trial number, were similar to the settings of the 5C-CPT training phase; that is, the houselight was always on, unless an omission, false alarm or inaccurate response was made; there was a 5 sec ITI; there was a timeout period of 5 sec; and following either an accurate response or a correct rejection, a tone was generated, a sugar pellet was released and the magazine light was switched on.

2.4. *Outcome measurement*

Response outcomes recorded by the ABETII were extracted and saved as a Microsoft Excel file. Outcome measurements on the probe test included: (1) ***motivation*** (number of trials completed out of 80 trials), (2) ***attention*** (*accuracy percentage* (# of correct responses/ (# of

correct + incorrect responses) *100) and *hit probability* (# of correct responses/ (# of correct+ incorrect+ omission responses) *100), (3) ***reaction time*** (correct response latency and latency to collect reward), (4) ***impulsivity*** (premature response percentage; # of premature response in go trials out of total completed trial *100), (5) ***learning*** (time taken to achieve each training session) and (6) ***cognitive flexibility*** (ability to maintain accuracy/hit probability despite varying stimulus duration).

Unfortunately, upon analysis, the sensitivity index (SI) and bias index (RI), as calculated according to (Young et al., 2009), of the 5C-CPT training phase revealed that animals did not learn the response inhibition portion of the task effectively enough to allow meaningful interpretation of the results.

Formula for SI and RI are as below:

$$SI = \frac{p(Hit) - p(FA)}{2[p(Hit) + p(FA)] - [p(Hit) + p(FA)]^2} ,$$

$$RI = \frac{p(Hit) + p(FA) - 1}{1 - [p(FA) - p(Hit)]^2} ,$$

with p (Hit) as hit probability, p (FA) as false alarm probability (a response in the no-go trial). SI provides a non-parametric assessment of sensitivity of the test (ranging from +1 to -1) with a value of 0 indicating an equally likely chance of animals to distinguish between a go trial and a no-go trial. The RI index, on the other hand, assesses animals' response bias or tendency to respond towards a signal stimulus versus a non-signal stimulus, with higher values (above 0) indicating a conservative response bias and lower values (below 0) a liberal response bias.

Although RI index analysis suggested an injury severity effect in the tendency to response to the stimulus at 1 month (rmTBI liberal bias response) and 12 months (msTBI conservative bias response) post injury (Figure 3), since our results showed that SI were not sensitive (values closer to 0, suggesting an equal probability of distinguishing signal and non-signal response; inhibition of non-signal response not achieved, as values are distant from +1) (Figure 3), the effect seen in the RI index becomes insignificant. Thus, data from the 5C-CPT

portion of the probe test (i.e. the No-Go trials) were omitted and only data from the 5CSRT portion of the probe test (i.e. the Go trials) results were included in the final data analysis.

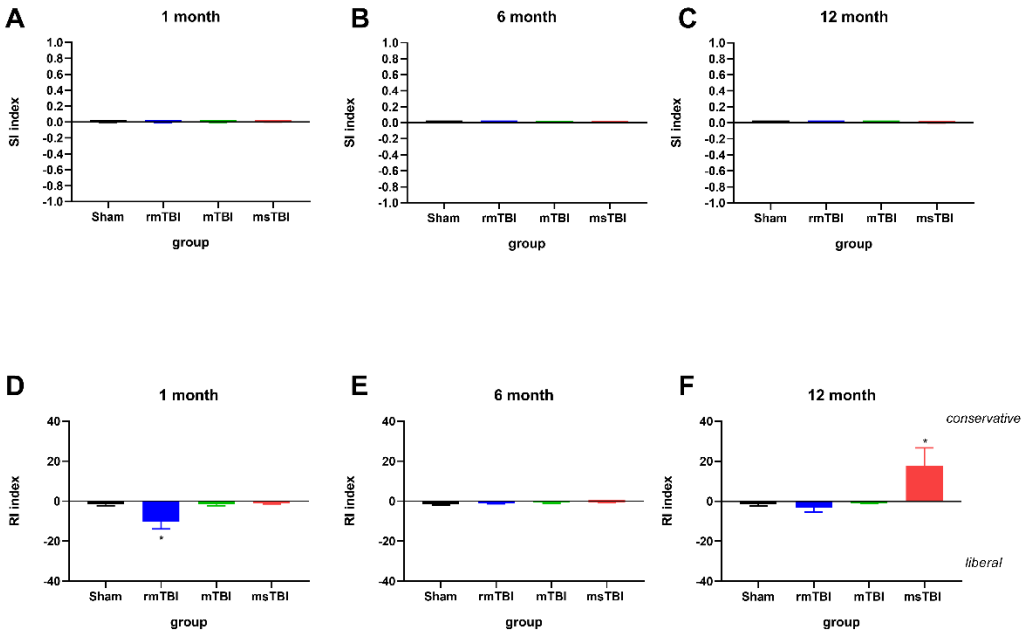

Figure 3: Sensitivity index (SI) and Response index (RI) as measured on the 5-Choice Continuous Performance task (5C-CPT), at A&D) 1 month, B&E) 6 months and C&F) 12 months post injury. Graphs represent mean ± SEM, (n=12-14; *p<0.05 compared to shams).

2.5. *Statistics*

Except where outlined, all data were analysed using IBM SPSS Statistics 24 and outliers were removed at 2 standard deviations from the mean value. A one-way analysis of variance (Bogdanova et al.) with group as the between subjects factor was performed at each follow-up timepoint to compare the effect of severity of injury on overall outcomes (i.e. accuracy percentage, hit probability, correct response latency, reward collection latency and premature response percentage) (Figure 6-8). A two-way repeated measures ANOVA with injury group and pre-/training sessions as between subject factors was performed for task repetition analysis in the pre-training and training phase (Figure 4). A two-way analysis of variance (Bogdanova et al.) was performed within each session of the training phase with injury group as one of the between subjects factor and follow-up timepoint as the other factor (Figure 4). A mixed effects

model was performed for each outcome (i.e. total trials, accuracy percentage, hit probability, correct response latency, reward collection latency and premature response percentage) with injury group and follow-up timepoint both as between groups factors (Figure 9). Additionally, a two-way ANOVA was also performed for the outcome hit probability, accuracy probability and omission probability, with injury group as one of the between subjects factor and stimulus duration as the other factor at each follow-up timepoint (Figure 10). Kruskal-Wallis test was performed for analysis of total trials completed (Figure 5) to compare injury severity effect on motivation at each timepoint. P values <0.05 were considered statistically significant.

3. Results:

3.1. *Learning during the training phase is dependent upon age of the animals, but not upon injury severity*

Learning was assessed based on the number of days required to reach criterion (80% in accuracy and 20% in omission out of 60 trials) at each stage of the training phase of 5CSRT. There was no significant effect of injury severity on days needed to reach criterion at any of the timepoints post-injury assessed (Figure 4A-C); 1 month ($F_{3,39}$=2.263, p=0.096), 6 months ($F_{3,42}$=0.155, p=0.926) and 12 months ($F_{3,37}$=1.504, p=0.229). However, we did observe a significant effect of training session number at each timepoint; 1 month ($F_{7,312}$=60.6, p<0.0001), 6 months ($F_{7,336}$=26.63, p<0.0001) and 12 months ($F_{7,\ 296}$=23.7, p<0.0001). A test of the interaction effect between injury severity and session number was insignificant at all timepoints (1 month ($F_{21,273}$=1.182, p=0.266), 6 months ($F_{21,294}$=0.768, p=0.758) and 12 months ($F_{21,259}$=0.614, p=0.907), indicating that animal performance within a given session did not vary as a function of initial severity of injury. As seen in Figure 4A-C, all follow-up timepoints post-injury show a similar pattern, where both the “must touch” task and session 6 pose a significant challenge to all groups, regardless of TBI severity, with more days required to pass criterion at these points compared to other sessions.

At 1 month post-injury, the “must touch” task required approximately 2 to 3 days more to acquire than the other tasks/sessions, with the exception of session 6. While in session 6, on the other hand, animals needed about 3-4 days more to pass criterion compared to other pre-/training session, excluding the “must touch” task. At 6 months, animals needed an additional 2 to 3 days to complete the “must touch” and session 6 tasks compared to other pre-/training sessions, with msTBI requiring up to 5 days to complete the session 6 task. However, at 12 months, only sham and rmTBI animals required 2 to 3 days of extra time to reach criterion at the “must touch” task compared to other sessions, excluding session 5 and 6.

Interestingly, we noticed that, while all animals acquired the task with relative ease up to session 4 (i.e. up to a 10 sec stimulus presentation duration), taking less than 2 days on average to reach criterion (Figure 4D-I), beginning at session 5, it took animals at later follow-up timepoints (12 months post-injury) longer to reach adequate levels of performance, regardless of injury group. In fact, within session 5 (Figure 4J), there were significant timepoint dependent learning difficulties ($F_{2,120}$=26.22, $p<0.0001$) in all groups at 12 months, especially when compared against 1 month animals (shams, p=0.012; rmTBI, $p<0.0001$; mTBI, p=0.0006 and msTBI, p=0.005). This suggests that age of the animal may affect learning capabilities in the later training sessions of the 5CSRT, as the stimulus duration becomes shorter (i.e. 5 sec duration or less). While significant differences between follow-up time points were not seen for any of the injury groups in session 6 (Figure 4K); ($F_{2,124}$=1.942, p=0.148), this may be a reflection of the difficulty of learning the task with a 2.5 sec stimulus duration.

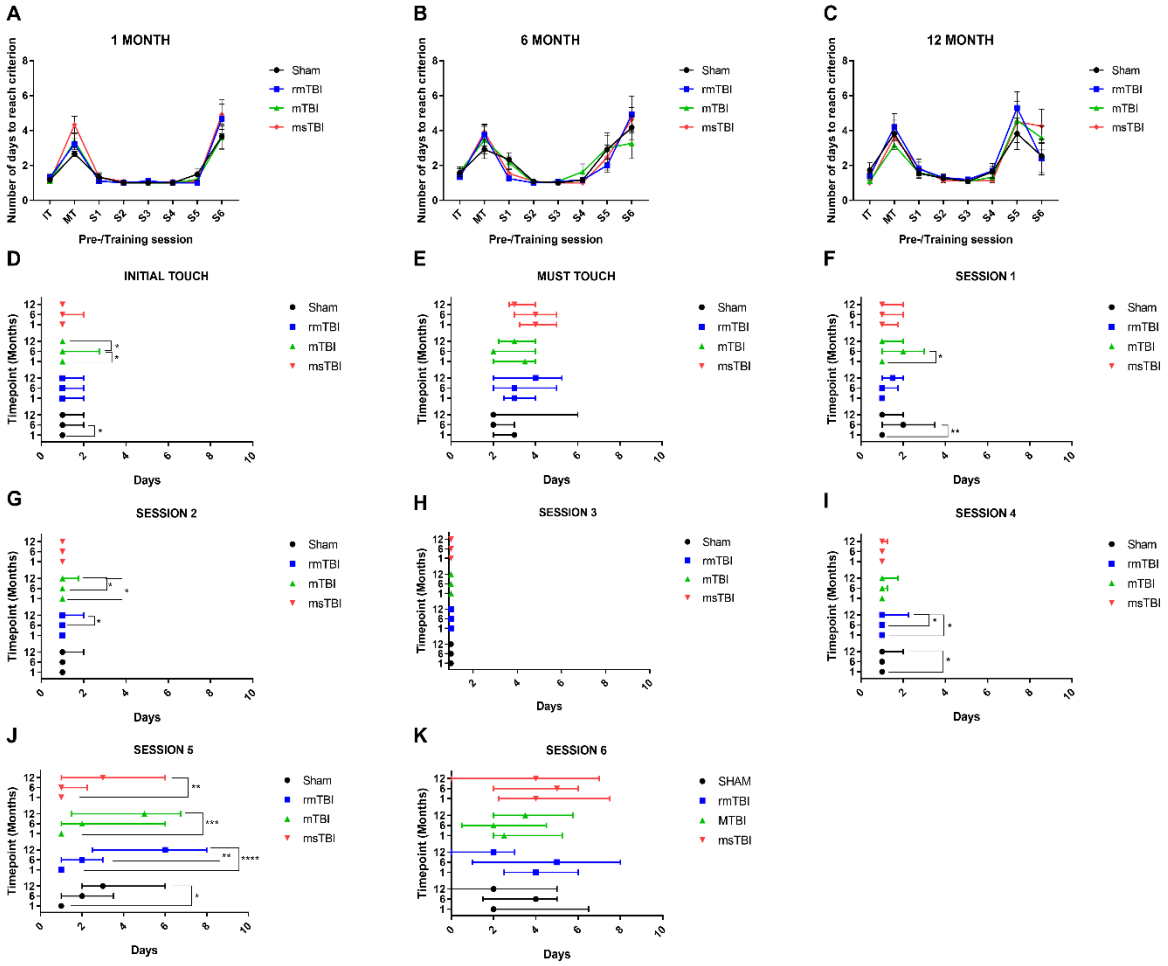

Figure 4: Number of days to complete the pre-training and training sessions by each injury groups at A) 1 month, B) 6 months and C) 12 months post injury. D-K) Number of days needed for completion separated by each pre-/training sessions with injury severity and follow-up timepoints as variable functions. Graphs represent A-C) mean ± SEM and D-K) median with interquartile range, (n=12-14, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared between follow-up timepoints).

3.2. *Motivation and executive function on probe day are not negatively impacted by TBI, regardless of the severity of the initiating injury*

Motivation and executive functions, including attention, reaction time and impulsivity, were tested through the 5CSRT on probe day. There was no significant effect of injury severity on motivation, as analysed by the total number of trials completed out of the 80 potential `go' trials (Figure 5), at any timepoint post-injury (1 month (median (range) of 77 (22-80) in sham, 71.5 (64-80) in rmTBI, 79.5 (40-80) in mTBI and 68 (51-80) in msTBI, $H_{4,42}$=3.569, p=0.312); 6 months (median (range) of 67 (38-80) in shams, 68 (42-80) in rmTBI, 53.5 (14-80) in mTBI and 60 (11-80) in msTBI, $H_{4,49}$=6.332, p=0.097); 12 months (median (range) of 66 (43-80) in shams, 62.5 (40-71) in rmTBI, 67.5 (37-80) in mTBI and 52 (22-80) in msTBI, $H_{4,44}$=6.301, p=0.098)).

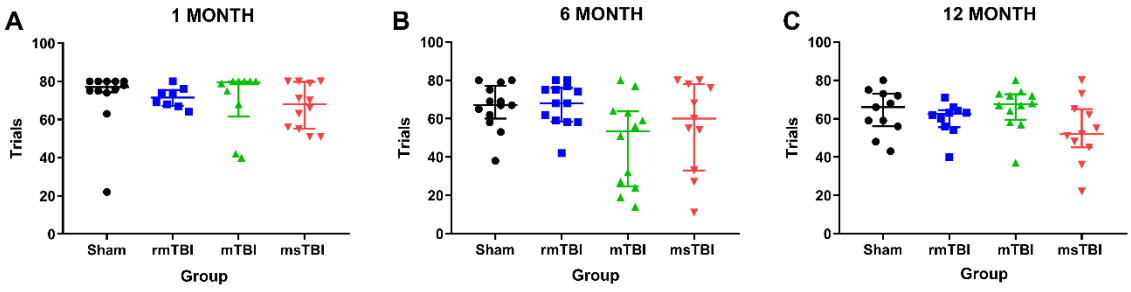

Figure 5: Motivation measured by total trials completed on probe test of the 5-choice serial reaction time task (5CSRT) at A) 1 month, B) 6 months and C) 12 months post injury. Graphs represent median with interquartile range and individual points, (n=12-14).

Attention was assessed by examining both hit probability (# of correct/ (# of correct+ incorrect+ omission responses) *100) and accuracy percentage (correct response/ (correct + incorrect response) *100). There was no effect of initial injury severity on hit probability at the 1 month (54.5%±5.91 in shams, 43.68%±7.43 in rmTBI, 54.51%±3.58 in mTBI and 48.44%±3.97 in msTBI, $F_{3,39}$=0.905, p=0.447), 6 months (46.47%±5.73 in shams, 40.54%±5.12 in rmTBI, 34.88%±8.21 in mTBI and 42.38%±8.32 in msTBI, $F_{3,45}$=0.502, p=0.683) or 12 months (32.96%±7.19 in shams, 27.79%±4.4 in rmTBI, 31.76%±4.31 in mTBI

and 19.15%±3.62 in msTBI, $F_{3,40}$=1.533, p=0.221) follow-up timepoints (Figure 6Ai-iii). Similarly, there were no significant differences in accuracy percentage (Figure 6Bi-iii) between any of the injury groups at 1 month (81%±2.15 in shams, 81.36%±3.29 in rmTBI, 77.34%±2.12 in mTBI and 73.63%±3.92 in msTBI, $F_{3,39}$=1.504, p=0.229), 6 months (82.44%±2.15 in shams, 78.65%±2.93 in rmTBI, 73.02%±7.62 in mTBI and 75.47%±5.62 in msTBI, $F_{3,45}$=0.705, p=0.554) or 12 months (78.84%±3.98 in shams, 79.49%±4.04 in rmTBI, 81.03%±3.67 in mTBI and 80.7%±3.63 in msTBI, $F_{3,40}$=0.073, p=0.974) post-TBI.

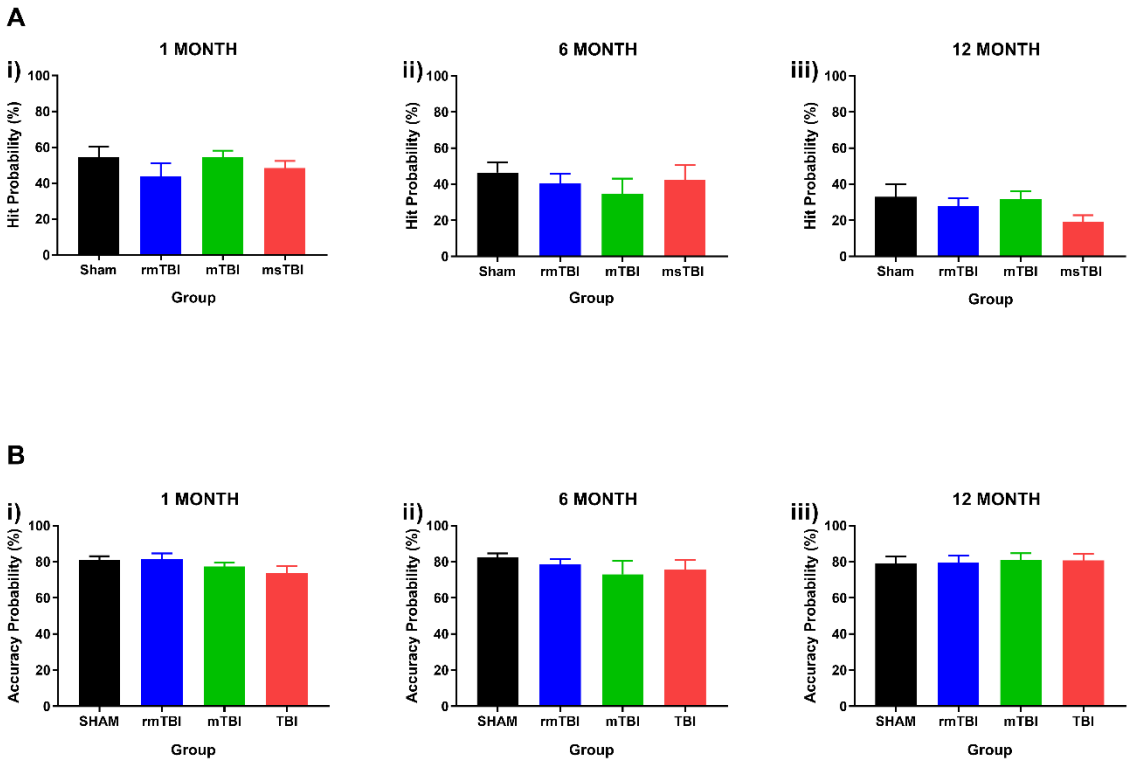

Figure 6: Attention assessed by Ai-Aiii) hit probability and Bi-Biii) accuracy probability at 1 month, 6 months and 12 months post injury on probe test of the 5-choice serial reaction time task (5CSRT). Graphs represent mean ± SEM, (n=12-14).

Reaction time was measured by both correct response latency (Figure 7Ai-iii) and reward collection latency (Figure 7Bi-iii). Neither of these variables showed any significant differences based on the initial TBI severity at 1 month (correct response latency: (1.24s±0.12 in shams, 1.07s±0.07 in rmTBI, 1.30s±0.1 in mTBI and 1.38s±0.07 in msTBI, $F_{3,37}$=1.686, p=0.187); reward collection latency: (1.31s±0.07 in shams, 1.35s±0.11 in rmTBI, 1.36s±0.09

in mTBI and 1.24s±0.05 in msTBI, $F_{3,38}$=0.458, p=0.714)), 6 months (correct response latency: (1.31s±0.07 in shams, 1.43s±0.09 in rmTBI, 1.74s±0.21 in mTBI and 1.65s±0.21 in msTBI, $F_{3,44}$=1.706, p=0.18); reward collection latency: (1.68s±0.09 in shams, 1.51s±0.08 in rmTBI, 2.03s±0.58 in mTBI and 1.83s±0.15 in msTBI, $F_{3,42}$=0.763, p=0.521)) or 12 months (correct response latency: (1.66s±0.18 in shams, 1.69s±0.13 in rmTBI, 1.69s±0.11 in mTBI and 1.97s±0.18 in msTBI, $F_{3,40}$=0.931, p=0.435); reward collection latency: (1.73s±0.17 in shams, 1.88s±0.11 in rmTBI, 1.82s±0.09 in mTBI and 2.12s±0.12 in msTBI, $F_{3,39}$=1.756, p=0.172)) post-injury.

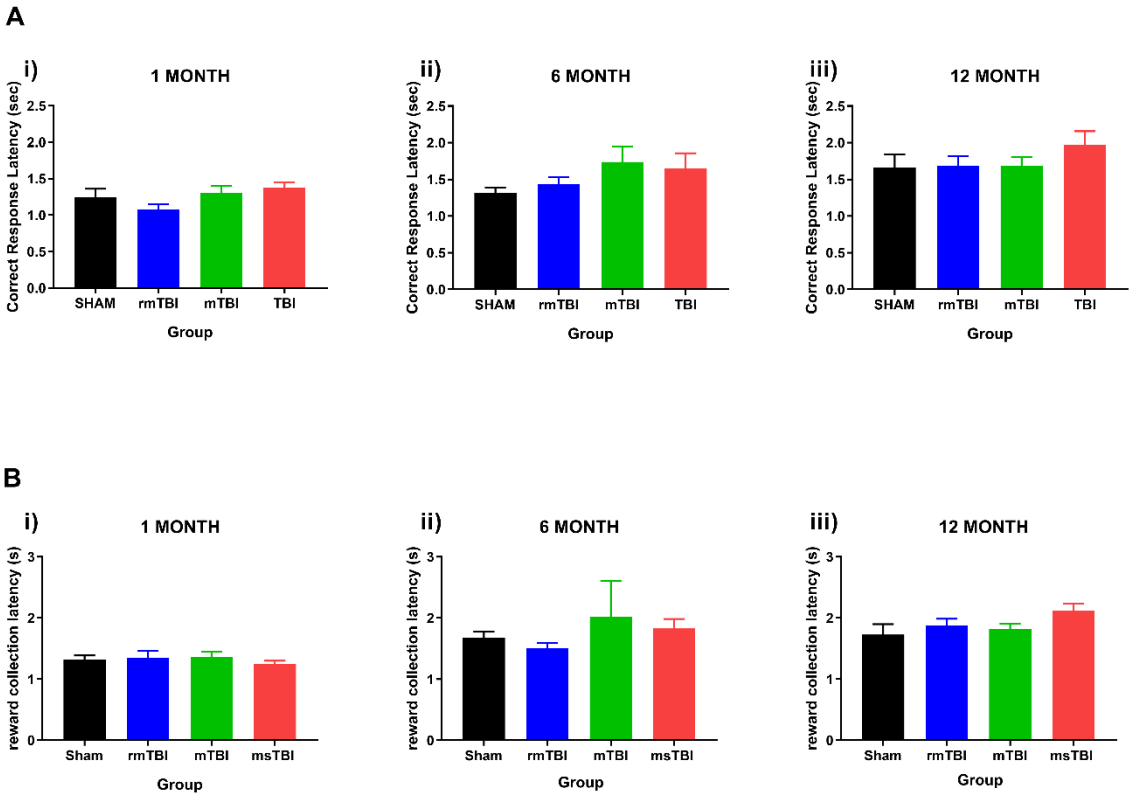

Figure 7: Reaction time assessed by Ai-Aiii) latency to make a correct response and Bi-Biii) latency to collect reward on probe test of the 5-choice serial reaction time task (5CSRT) at 1 month, 6 months and 12 months post injury. Graphs represent mean ± SEM, (n=12-14).

Impulsivity was assessed by looking at the percentage of premature responses in the go trial out of the total number of go trials completed. A premature response was defined as a response that occurred during the 5 sec ITI. There were no significant differences in percentage

of premature responses based on initial injury severity (Figure 8) at 1 month (11.31%±2.65 in shams, 18.44%±5.67 in rmTBI, 22.7%±10.28 in mTBI and 23.78%±6.68 in msTBI, $F_{3,39}$=0.783, p=0.511), 6 months (11.73%±2.64 in shams, 10.01%±2.15 in rmTBI , 15.17%±4.52 in mTBI and 7.92%±2.18 in msTBI, $F_{3,43}$=0.939, p=0.43) or 12 months post injury (11.68%±2.2 in shams, 8.47%±2.01 in rmTBI, 9.63%±2.51 in mTBI and 4.42%±1.57 in msTBI, $F_{3.40}$=2.067, p=0.12).

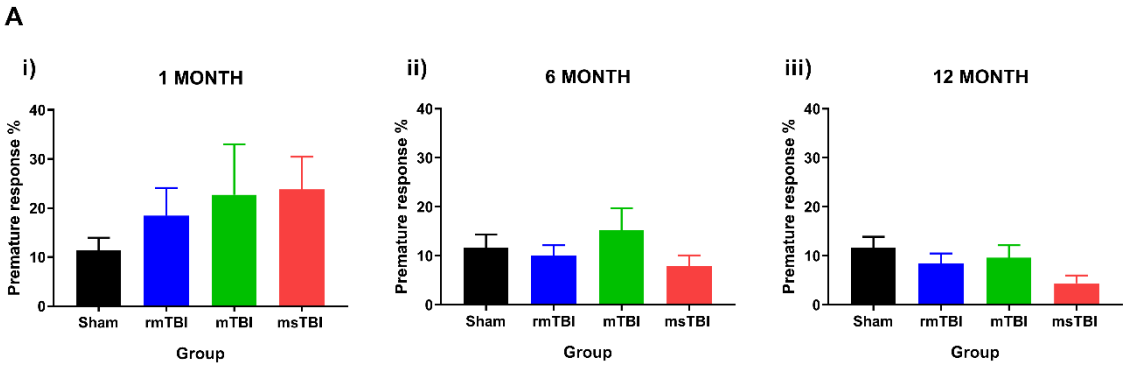

Figure 8: Impulsivity assessed by percentage of premature response made on probe test of the 5-choice serial reaction time task (5CSRT) at Ai) 1 month, Aii) 6 months and Aiii) 12 months post injury. Graphs represent mean ± SEM, (n=12-14).

3.3. *Motivation, attention and reaction time are negatively affected by age on probe day, while impulsivity may improve over time*

While there were no effects of TBI on motivation or executive function as measured by task performance at 1 month, 6 months or 12 months post-injury, there was a significant effect of the age of the animal at testing on many of these variables (Figure 9), with the exception of accuracy probability ($F_{1.914,118.7}$=0.426, p=0.646; Figure 9C). Motivation ($F_{1.891,115.4}$=8.882, p=0.0003), hit probability ($F_{1.807,112}$=13.86, p<0.0001) and reaction time (as measured by both correct response latency; $F_{1.776,108.4}$=10.29, p=0.0002 and reward collection latency; $F_{1.431,53.67}$=10.61, p=0.0006) all showed significant impairment with age, while impulsivity appeared to improve ($F_{1.379,52.41}$=6.577, p=0.0072);

Interestingly, the same pattern of effects was not seen in all injury groups. Despite this, a significant interaction between injury severity and follow-up timepoint was not seen for any of the outcome variables: hit probability ($F_{6,124}$=0.701, p=0.649), accuracy percentage ($F_{6,124}$=0.56, p=0.761), premature response ($F_{6,123}$=1.081, p=0.378), reward collection latency ($F_{6,121}$=0.725, p=0.631) or correct response latency ($F_{6,122}$=0.89, p=0.505), although there was a trend towards significance for motivation ($F_{6,122}$=2.11, p=0.06). This indicates that age-related changes in executive function did not differ as a function of original injury severity.

Post hoc analysis of the number of trials completed at each timepoint showed that sham and rmTBI animals had a significant decrease in the number of trials completed at 12 months post injury when compared to 1 month post injury (median of 66 trials in shams and 62.5 trials in rmTBI at 12 months vs median of 77 trials in shams and 71.5 trials in rmTBI at 1 month, p=0.04 in shams and p=0.02) (Figure 9A). For hit probability, there were decreases in the shams (32.96% ±7.19 vs 54.5%±5.91, p=0.056), mTBI (31.76%±4.31 vs 54.51%±3.58, p=0.011) and msTBI (19.15%±3.62 vs 48.44%±3.97, p=0.002) groups at 12 months when compared against 1 month post injury (Figure 9B). A significant difference in hit probability between these timepoints was not seen in the rmTBI group (27.79%±4.40 vs 43.68%±7.43 at 1 month, p =0.301). In regards to reaction time, msTBI animals showed significantly longer latency at 12 months when compared to 1 month post injury for reward collection (2.12secs ±0.118 vs 1.24secs ±0.054, p<0.0001; Figure 9E), with a trend of increased response latency to correctly respond to the stimulus (1.97secs ±0.183 vs 1.38secs ±0.072, p=0.06; Figure 9F). In contrast, in comparison to animals at 1 month post-injury, at 12 months following injury, mTBI animals only showed increases in reaction time to collect the reward (1.82secs ±0.093 vs 1.36secs ±0.087, p=0.003), while rmTBI animals had significantly increased correct response latency (1.69secs ±0.13 vs 1.07secs ±0.074, p=0.024) as well as reward collection latency (1.88s ±0.11 vs 1.35s ±0.11, p=0.035). Changes in reaction time for either variable were not seen in sham animals at these timepoints. For impulsivity, while there was a significant main effect test of follow-up time on impulsivity, post hoc analysis only revealed a trend of decreased impulsivity

in msTBI animals over time (23.78%±6.68 at 1 month vs 7.92%±2.18 at 6 months (p=0.059) and 4.42%±1.57 at 12 months (p=0.06)).

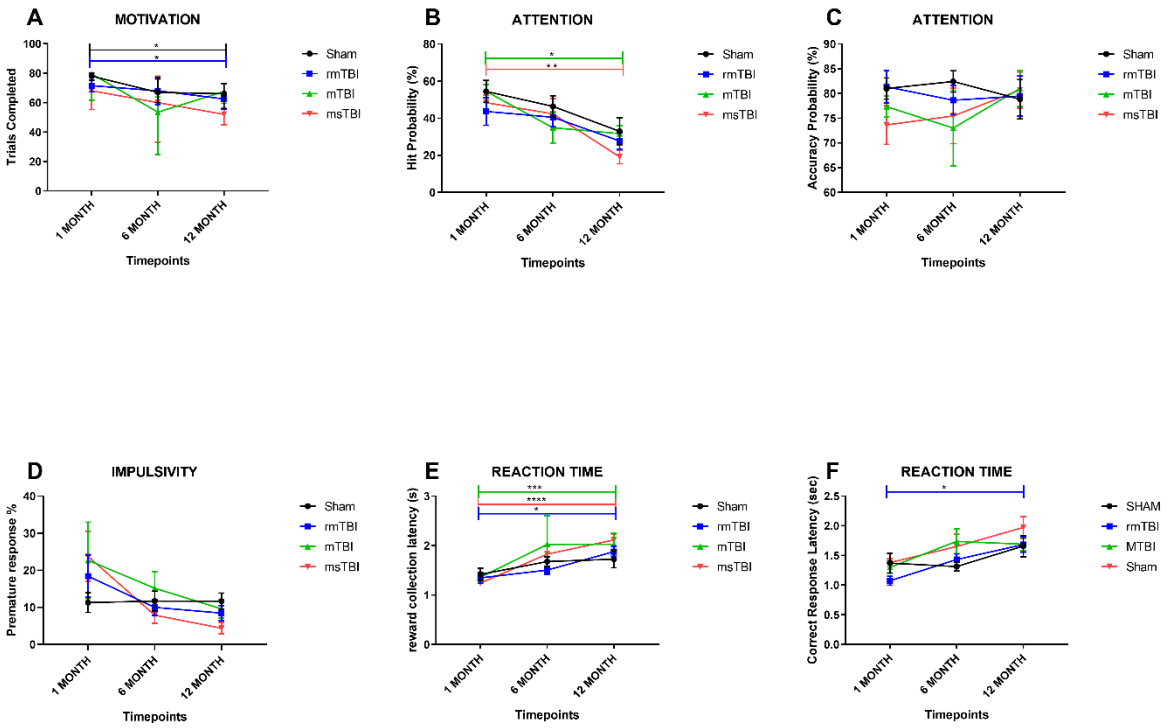

Figure 9: Facets of executive function, A) Motivation, B-C) Attention, D) Impulsivity and E-F) Reaction time, measured on probe test of the 5-choice serial reaction time task (5CSRT) with injury severity and follow-up timepoints (age) as variable functions. Graphs represent mean ± SEM, (n=12-14, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared between follow-up timepoints).

3.4. *Cognitive flexibility might be affected by injury severity at chronic stages post-injury*

Cognitive flexibility was measured on probe day using hit and accuracy probability for varied stimulus presentation durations within the 80 trials. After achieving 80% hit probability and accuracy during the training phase, with a stimulus presentation duration of 2.5 secs, on probe day, animals had to switch their attention between the varying stimulus presentation durations (i.e. 0.5, 1, 2 ,3 and 4 secs) in each trial, in order to achieve similar hit and accuracy probability. As expected, there was an effect of stimulus duration at all follow-up timepoints on both hit probability (1 month ($F_{4,152}$=63.79, p<0.0001), 6 months ($F_{4,180}$= 41.2, p<0.0001) and 12

months ($F_{4,160}$= 35.58, p<0.0001)) and accuracy probability (1 month ($F_{4.152}$=33.4, p<0.0001), 6 months ($F_{4,180}$=18.15, p<0.0001) and 12 months ($F_{4,160}$=12.28, p<0.0001)), with both probabilities decreasing significantly with reducing stimulus duration. There was no significant interaction at any follow-up timepoint between stimulus duration and injury group for either hit probability (1 month ($F_{12,152}$=0.844, p=0.606), 6 months ($F_{12,180}$=0.597, p=0.843) and 12 months ($F_{12,160}$=0.823, p=0.627)) or accuracy probability (1 month ($F_{12,152}$=0.633, p=0.811), 6 months ($F_{12,180}$=0.234, p=0.996) and 12 months ($F_{12,160}$=0.972, p=0.478)), indicating that impairments in performance seen with reduced stimulus duration did not vary as a function of the initiating injury.

Our results also showed no significant effect of injury on hit probability at any stimulus duration (Figure 10A-C) at 1 month ($F_{3,38}$=1.789, p=0.166), 6 months ($F_{3,45}$=0.464, p=0.709) or 12 months ($F_{3,40}$=1.476, p=0.236) post-injury. Similarly, there was no effect of injury on accuracy probability, when measured at varying stimulus durations, at either 1 month ($F_{3,38}$=1.248, p=0.306; Figure 10D) or 6 months ($F_{3,45}$=1.962, p=0.133; Figure 10E) post-injury. There was, however, a significant effect of injury at 12 months post injury ($F_{3,40}$=3.221, p=0.033; Figure 10F). Post-hoc analysis indicated an effect for the 2 sec duration only, with moderate-severe TBI animals exhibiting less accuracy than shams (52.07% ±15.22 vs 84.34% ±8.91, p=0.083), rmTBI (52.07% ±15.22 vs 87.78% ±6.51, p=0.052) and mTBI (52.07% ±15.22 vs 90.09% ±4.43, p=0.023) animals.

Finally, we investigated omission percentage (that is, the percentage of trials in which the animal did not make a response either immediately after stimulus presentation or during the limited hold time) at the various stimulus durations. While the number of omissions did decline significantly with increasing stimulus duration at all follow-up time points assessed (1 month ($F_{4,152}$=23.61, p<0.0001), 6 months ($F_{4,180}$=21.09, p<0.0001) and 12 months ($F_{4,160}$=27.96, p<0.0001)), there was no effect of injury at any of these points (1 month ($F_{3,38}$= 1.335, p=0.277), 6 months ($F_{3,45}$= 0.418, p=0.741) and 12 months ($F_{3,40}$= 1.188, p=0.327) (Figure 10G-I). Similarly, there was not a significant interaction between injury severity and stimulus duration

at any follow-up timepoint, indicating that the reduction in omission errors seen with longer stimulus durations was not dependent on the initial severity of injury (1 month ($F_{12,152}$=0.678, p=0.771), 6 months ($F_{12,180}$=0.399, p=0.963) and 12 months ($F_{12,160}$=0.829, p=0.620)).

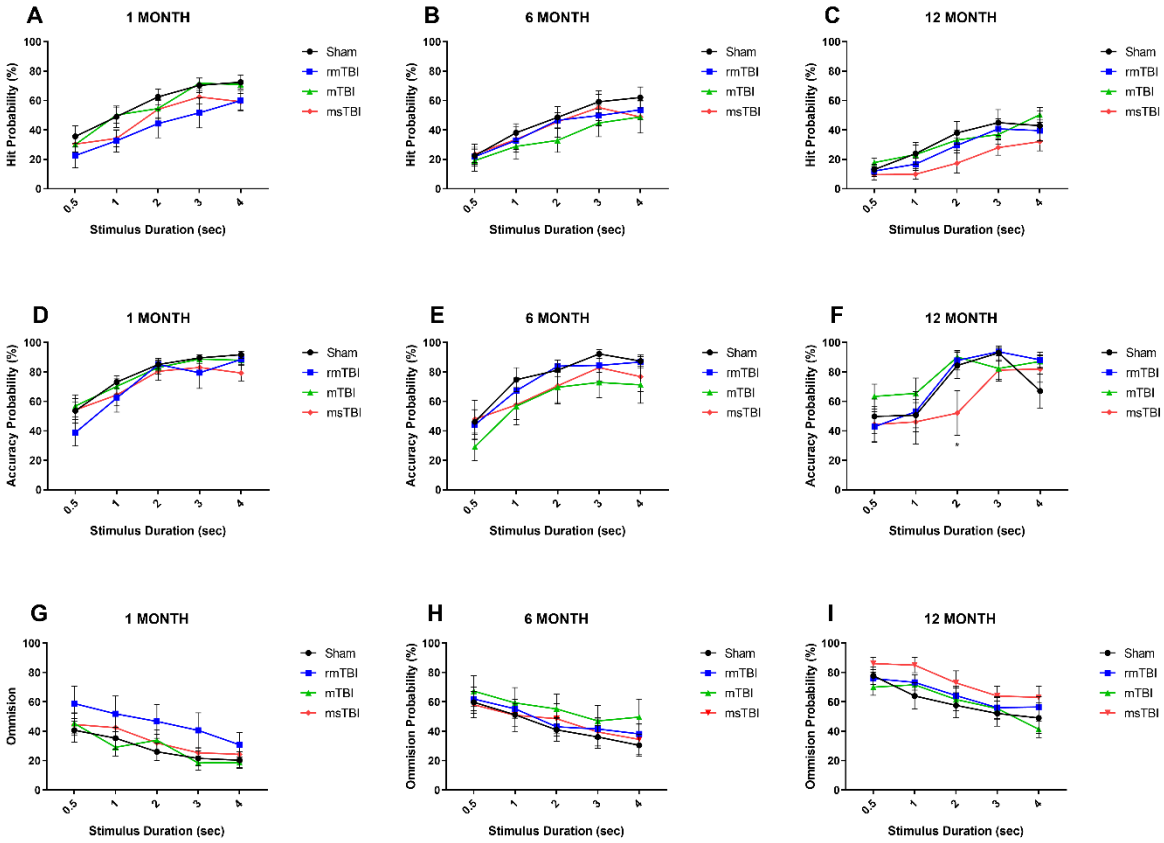

Figure 10: Attention with injury severity and stimulus duration as variable functions were assessed by A-C) hit probability, D-F) accuracy probability and G-I) omission probability, at 1 month, 6 months and 12 months post injury, on probe test of the 5-choice serial reaction time task (5CSRT). Graphs represent mean ± SEM, (n=12-14, *p<0.05 compared to shams).

4. Discussion:

This study aimed to investigate the temporal profile, up to 12 months post-injury, of the evolution of executive dysfunction following different severities of injury in an experimental model of DAI. In order to do so, we utilised a rodent touchscreen paradigm to administer the 5C-CPT, an extension of the 5CSRT. The touchscreen 5CSRT task functions similarly to the non-computerised 5CSRT model, first developed for use with rodents in the 1980s (Carli et al.,

1983), which requires animals to nose-poke into one out of five apertures when a stimulus light is presented pseudo-randomly above it. A number of executive functions can be assessed using this task, including attention (response accuracy), inhibitory control (premature responses) and processing speed (response and reward collection latency). Going even further, the 5C-CPT increases the difficulty of the task by requiring animals to be vigilant and discriminate between signal and non-signal (noise) stimuli (Young et al., 2009), with animals rewarded both for responding to certain pre-set stimuli and for not responding to other pre-set stimuli, providing further insight into inhibitory control (Mar et al., 2013b). Interestingly, our results indicate that, while there was a significant effect of age at follow-up on many of the variables investigated, there was minimal effect of initial injury severity on learning, motivation or any of the components of executive function evaluated, including attention, reaction time or impulsivity.

Within our study, the largest effect on task performance was due to age. Up to session 4 (i.e. up to a 10 sec stimulus presentation duration), all animals were able to learn the task, regardless of age post-injury, with relative ease, taking less than 2 days on average to reach criterion. As the stimulus duration became shorter, however, it took older animals (i.e. those at 6 months and 12 months post-injury) longer to reach adequate levels of performance, regardless of injury group, when compared to younger animals. This is consistent with literature showing age-related changes in visual processing, with older adults performing significantly slower than younger adults on both face and location matching tasks (Grady et al., 1994). In fact, one of the leading theories of cognitive ageing is the processing speed hypothesis, which states that declines in the ability to process information rapidly due to increased age lead to impairments in the ability to perform higher-order cognitive tasks (Salthouse, 1996). During the 5CSRT task, as stimulus duration decreased, the perceptual signal was weakened, thereby increasing cognitive load and requiring more cognitive resources to interpret the signal, compromising cognitive performance for the older animals (Schneider and Pichora-Fuller, 2000; Zekveld et al., 2011).

Such declines in performance are most prominent from the age of 45 (Hoyer et al., 2004), which is consistent with the middle-age of the rodents used in the current study. In support of this, in a cohort with a mean age of just under 54 years, a lower accuracy hit rate was observed on the Rapid Visual Information Processing task compared to those reported in earlier studies utilising this task in younger cohorts, suggesting detectable declines in visual processing that may impact performance even from middle age (Neale et al., 2015). Similarly, in a previous study utilising the standard 5CSRT task in rats, middle-aged (i.e. 15 month old) animals were significantly slower to learn the task than young (i.e. 3 month old) rats, an effect that was exacerbated in aged (i.e. 22 month old rats), a subset of whom could not reach criterion at a 0.5 sec stimulus duration (Jones et al., 1995). This suggests that age of the animal may affect learning capabilities in the later training sessions of the 5CSRT, as the stimulus duration becomes shorter (i.e. 5 sec duration or less). While significant differences between follow-up time points were not seen for any of the injury groups in session 6, this may be a reflection of the difficulty of the task with a 2.5 sec stimulus duration, a conclusion which is further supported by the significant variability seen in the acquisition of this task.

Age also appeared to have a significant effect on 5CSRT performance on probe day, following task acquisition. Rats showed significant impairment on measures of motivation (number of trials completed), attention (hit probability) and reaction time, as well as possible (trend) decrease in impulsivity (number of premature responses). Interestingly, however, variables appeared to be differentially affected based on the initial severity of injury. Most consistently, as might be expected in a test that is particularly sensitive for detecting attentional differences in rodents (Bhandari et al., 2016; Fizet et al., 2016; Higgins and Silenieks, 2017) attention was affected in both the mTBI and msTBI groups, with hit probability lower in animals at 12month follow-up compared to 1 month follow-up. While not significant, animals in the rmTBI also showed a decline in hit probability between groups at the 1 month and 12 month follow-up timepoints. Such impairment is consistent with the extensive literature on declines in attention that occur even in healthy ageing (for review, see (Kallus et al., 2005)).

These declines are often attributed to deficits in inhibitory control that occur with increased age, leading older adults to have an impaired ability to inhibit the processing of task-irrelevant, distracting information (Lustig et al., 2007).

Attentional impairments may be detectable even from middle-age, with studies indicating that age-related change in the executive attention network may be detectable from the fourth decade of life (Zhou et al., 2011). This is consistent with results from a study in rats by Guidi and colleagues (2015), using the 5CSRT, which demonstrated deficits in attention and executive function, as measured by a decrease in choice accuracy, an increase in the number of omissions and an increased response latency, in middle-age animals (10-14 months of age) compared to young animals (3-6 months of age) (Guidi et al., 2015). Similarly, in another study, both middle aged (15 months) and aged rats (22 months) were slower to make a correct response, but not to collect the reward, compared to young animals (3 months) (Jones et al., 1995). In contrast, in our study, increases in time to make a correct response were increased between 1 and 12-months post-injury in the rmTBI, mTBI and msTBI groups; conversely, increases in time to collect a reward was only seen in rmTBI groups. Sham animals were unaffected on both variables, although this may be a reflection of strength of acquisition of the task, with hit probabilities of less than 60% in all groups in the 1-month follow-up group, dropping to less than 40% in the 12-month follow-up group. It is possible that with an altered training paradigm, leading to stronger task acquisition, we may be better equipped to detect alterations in task performance.

Surprisingly, in the current study, there were no differences between injury groups for task acquisition, motivation, or any of the executive function variables examined, including attention, reaction time or impulsivity. The only detectable difference dependent on injury severity was a significant effect of injury on accuracy probability at 12 months post injury, with msTBI animals exhibiting reduced accuracy compared to all other groups, but only for the 2 sec stimulus duration. This appears to be a measure of the lower limit of threshold detection, with animals at the 12 month follow-up timepoint not able to perform the task at either the 0.5

sec or 1 sec stimulus presentations, as evidenced by the low hit probability (less than 20%) and the high omission probability (approximately 80%) at these durations. While animals in the other groups appear better able to perform the task at the 2 sec stimulus duration (as evidenced by an increased hit probability and reduced omission probability), as might be expected given the similarity of this stimulus duration to the 2.5 sec stimulus used in session 6 of the training paradigm, msTBI animals remain unable to perform the task until a longer stimulus duration (i.e. 3 sec or longer) is given, at which point their performance approaches the level of the other groups. This may suggest that age exacerbates the subtle injury impairments seen following moderate-severe TBI, although additional studies, using older animals, will be needed in order to more fully probe this effect.

The lack of injury effect was particularly surprising given that previous studies in the CCI model have detected changes in executive function at sub-chronic timepoints ranging from 4 weeks to 11 months injury (Bondi et al., 2014; Chou et al., 2016a; Njoku et al., 2019; Shaver et al., 2019a; Vonder Haar et al., 2016; Vonder Haar et al., 2017) for review, see (Ozga et al., 2018). For example, using the attentional set-shifting task, which is analogous to the Wisconsin Card Sorting Test, deficits in executive function and behavioural flexibility were found up to 4 weeks post-TBI and were more pronounced as a function of injury severity (Bondi et al., 2014; Njoku et al., 2019). Similarly, using the Rodent Gambling Task, an analogue of the Iowa Gambling task, impairments in decision-making ability were detected up to 12 weeks after moderate-severe injury (Shaver et al., 2019a). Deficits in executive function, as indicated by impairments in reversal learning on a rule shift assay, have been observed up to 5.5 months post-injury in a CCI model that produced moderate-severe frontal contusions (Chou et al., 2016a). At an even longer follow-up timepoint, performance on a differential reinforcement of low-rate responding schedule of reinforcement was impaired up to 11 months following a severe bilateral focal injury to the frontal cortex (Lindner et al., 1998). Interestingly, such executive function deficits may not be “dose-dependent”, as neither impulsive decision making

(Vonder Haar et al., 2017) nor response inhibition deficits (Vonder Haar et al., 2016) differed as a function of initial injury severity.

It is important to note, however, that almost all of the previous studies of long-term executive function alterations following TBI in rodents have used models of injury that produce a focal injury. Such "pure" forms of focal injury occur in only 28% of moderate-severe TBI cases, while diffuse axonal injury is seen in 72% of individuals, with "pure" diffuse axonal injury associated with significantly lower scores on the Glasgow Coma Scale (Skandsen et al., 2010). Acutely, animals injured via fluid percussion (FPI), which produces a mixed focal and diffuse injury, have been shown to display working memory deficits, as assessed by a T-maze task, up to one week post-TBI, an effect that was accompanied by alterations in prefrontal cortex function (Smith et al., 2015b). More chronically, while animals injured via FPI have been shown to have persistent cognitive deficits at 12 months post-injury (Hausser et al., 2018; Sell et al., 2017), executive function has not been specifically assessed in this model. Similarly, while recent work from our lab in the same model of DAI used in the current study has found subtle deficits in cognitive flexibility at both 3 months (Arulsamy et al., 2018b) and 12 months (Arulsamy et al., 2019) following msTBI, the evolution of change in executive function had not previously been assessed following diffuse axonal injury. While the assessment of several key executive function variables, such as attention and impulsivity, was therefore a significant step forward in the current study, we were still unable to assess response inhibition, as rats failed to meet criteria, as measured by either the SI or RI (Young et al., 2009), for having learned the 5C-CPT task sufficiently to allow for meaningful interpretation of the results. Thus, we were unable to assess data from No-Go trials of the 5C-CPT, which would have allowed us to evaluate this important component of executive function. Future studies should increase the length of training on the 5C-CPT in order to improve the likelihood of animals acquiring the task. Such adjustments may be particularly important for older animals, or for animals who have suffered a significant brain injury.

Despite the lack of significant injury effect, this is still the first study to investigate executive function change using the rodent touchscreen platform in an experimental model of TBI. In fact, to date, to the best of our knowledge, only one other study has conducted cognitive testing using the touchscreen platform following TBI (Robinson et al., 2018). That study used the platform to assess visual discrimination at 90 days following an early life CCI at postnatal day 12, but did not assess executive function (Robinson et al., 2018). Given the older age of our rats (i.e. 14-15 months), it is important that future studies take the age of the animal into consideration when optimising the touchscreen platform for long-term studies in rats, due to the decrease in reaction speed as the animal ages, which may mask important cognitive effects, such as impulsivity. Therefore, in studies with older cohorts of animals, the limited hold (time held for a response to be made) after stimulus presentation and ITI (time between trials) may need to be increased, so that all executive function variables may be measured consistently across different ages of animals. Additionally, it may be important to take into account the strain of rat used for studies. Sprague Dawley (SD) rats are albino and have much lower visual acuity than non-albino strains (Prusky et al., 2002). Kumar and colleagues (2015) suggested that low visual acuity in albino SD rats may prevent them from performing effectively on the touchscreen platform (Kumar et al., 2015a). Conversely, previous work from Tim Bussey's group (2008) has shown that SD rats perform as well as Lister Hooded rats, with no difference between strains either on percent of correct responses or on number of sessions required to meet criterion (Bussey et al.). Despite this, he does acknowledge that sensitive rats, which may be the case with older SD rats, may be more responsive towards aversive stimuli rather than appetitive. Thus, significant future work is needed in order to optimise the touchscreen platform for sophisticated cognitive testing in models of ageing and experimental TBI, particularly those requiring long-term follow-up timepoints.

Taken together, data from the current study suggest that, while age at follow-up significantly impacts upon several facets of executive function, TBI does not, regardless of initial injury severity. However, there are a number of caveats, including potential insufficient

length of follow-up and limitations to the behavioural training protocol used, that must be taken into account when evaluating these results. Despite the limitations, we believe that, with further optimisation, the touchscreen cognitive chamber could be a sensitive, sophisticated and reliable testing paradigm for assessing alterations in executive function following TBI. Given the prevalence, persistence and significance of executive function impairments following TBI, the development and optimisation of such a task to better investigate the evolution of this impairment following different severities of injury is critically important.

Chapter 5
Subtle molecular pathology in motor-related regions at one year post mild diffuse traumatic brain injury; a possible link to motor neurone disease

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Alina Arulsamy		
Contribution to the Paper	Performed analysis of molecular data, interpreted data and wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/02/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript editing		
Signature		Date	26/2/19

Please cut and paste additional co-author panels here as required.

Subtle molecular pathology in motor-related regions at one year post mild diffuse traumatic brain injury; a possible link to motor neurone disease

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AA was involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

Abstract

Traumatic brain injury (TBI) is more than just an acute injury but a risk factor for later development of neurodegenerative diseases. Secondary injury mechanisms after the initial insult may cause neurodegenerative-like abnormal protein accumulations such as tauopathy, alpha-synuclein inclusions and TDP-43 mis-localization and phosphorylation within the brain. While these pathological proteins are usually evident in post-mortem TBI patients, depending on the nature and severity of TBI, the neuropathological link between experimental TBI and neurodegeneration is still debatable as very few preclinical studies have investigated this relationship chronically (beyond 6 months). Thus, this study aimed to determine the neuropathological changes at a molecular level, particularly in neurodegenerative markers, at 12 months post diffuse TBI of varied severity. Male Sprague Dawleys (10-12 weeks old) of 420-450g were either subjected to sham surgery (n=6) or the Marmarou's impact acceleration model of diffuse injury for a single mild TBI (n=6), repetitive mild TBI (n=7) or moderate to severe TBI (n=7). At 12 months following injury, animals were perfused, and molecular analysis were performed on their brain and spinal cord samples. Our results showed increases in cytoplasmic mis-localization of TDP-43 proteins in the cervical spinal region of the single and repetitive mild TBI groups. Moreover, abnormal changes in NeuN and phosphorylated-TDP-43 levels were also found in motor cortex and spinal regions of the single and repetitive mild TBI animals at 12 months post injury. There was a lack of neuropathological changes in the prefrontal cortex, hippocampus, substantia nigra and striatum of all groups. Thus, our study suggest mild diffuse TBI may have higher implications towards motor neurone diseases such as ALS compared to other neurodegenerative diseases at 12 months post injury. Further timepoint studies (18 months) may ensure the progression of this neuropathology into muscle atrophy post injury and possibly the emergence of other neurodegenerative disease pathology as well.

5. Introduction

Annually 50 to 60 million people are hospitalised for traumatic brain injury (TBI) worldwide. Billions of dollars are spent yearly on TBI associated cases, creating a huge economic burden in countries like the United State of America ((US), 2003) and Australia (Helps et al., 2008). Moreover, this economic burden is set to double in the future, as TBI is not just an acute injury (Masel and DeWitt, 2010b), but can increase the risk of later developing neurodegenerative diseases like Parkinson's Disease (PD), Alzheimer's Disease and Amyotrophic Lateral Sclerosis (ALS) (Chen et al., 2007b; Faden and Loane, 2015; Gardner et al., 2015a; Nemetz et al., 1999a; Omalu et al., 2005; Plassman et al., 2000; Sundman et al., 2014a). Indeed, population-based studies have found hazard risk ratios of 1.1-1.7 for dementia (Fleminger et al., 2003b; Gardner et al., 2014b; Wang et al., 2012), 1.57 for PD (Jafari et al., 2013) and 1.7-3.2 for ALS (Chen et al., 2007b) following TBI, which increases with the severity of the injury (Nordstrom and Nordstrom, 2018; Tolppanen et al., 2017).

A cascade of secondary insults which include a range of biochemical and cellular changes such as neuroinflammation, oxidative stress, mitochondrial dysfunction and other pathological changes, may persist or emerge years later post TBI (Corrigan et al., 2016b; Sivanandam and Thakur, 2012). These secondary pathologies may stem from the primary insult of axonal tearing and shearing that occurs following a purely diffuse TBI (Corrigan et al., 2016b), creating widespread neuronal damage within multiple vulnerable areas of the brain. This is thought to promote the development of intracellular aberrant protein aggregates, a neuropathological feature of neurodegenerative disease. These aggregates are thought to drive neurodegeneration leading to the onset of disease. Cytoplasmic proteins including tau in Alzheimer's disease, TDP-43 in ALS and α-synuclein in PD form insoluble inclusions within neurons. These proteins are typically abnormally hyperphosphorylated facilitating their aggregation. Indeed post mortem and imaging studies have found tauopathy (Johnson et al., 2012b; Tagge et al., 2018), amyloid plaques (Johnson et al., 2012b; Scott et al., 2016), TDP-43 proteinopathy (Johnson et

al., 2011a; Yang et al., 2014) as well as alpha-synuclein inclusions (Mondello et al., 2013; Uryu et al., 2007) in TBI patients, where imaging studies revealed these neuropathology to be evident as early as 24 hours post injury and may persist up to 6 months post injury (Mondello et al., 2013; Tagge et al., 2018).

The presence of these abnormal proteins associated with neurodegenerative disease have also been observed in pre-clinical models of TBI. Tan *et al* (2018) and Wright *et al* (2017) witnessed that rodents had increased phosphorylation and cytoplasmic mis-localisation of TDP-43 proteins as early as 24 hours post TBI (Tan et al., 2018) which persisted and exacerbated into motor neuronal damage at 12 weeks post injury (Wright et al., 2017b). In addition, Wright *et al* (2017) showed that TDP-43 abnormalities may precede and drive motor neuronal changes, particularly neuronal loss and atrophy which attributes to the motor impairment following fluid percussion injury (Wright et al., 2017b). This is also true for other neurodegenerative markers such as alpha-synuclein which is found overexpressed in the substantia nigra (Gilbert et al., 2014) of rodents at 60 days post controlled cortical impact injury, elucidating the link towards Parkinson's disease in a preclinical TBI model (Acosta et al., 2015b).

However, only a handful of these pre-clinical studies have examined protein accumulation post-TBI chronically after diffuse injury (Arulsamy et al., 2018a; Chen et al., 2004). Furthermore, chronic studies to date often concludes at 1 to 6 months post injury (Bramlett et al., 1997; Chen et al., 2004; Gao et al., 2017; Laurer et al., 2001; Shultz et al., 2015), rarely investigating further timepoints (Mouzon et al., 2014; Pierce et al., 1998). This may not accurately translate to human studies as neurodegeneration in TBI patients only becomes evident at late stages of life. Moreover, with more studies opting for focal injury model (Bramlett et al., 1997; Pierce et al., 1998; Shultz et al., 2015), there is a need for studies to investigate the more clinically relevant diffuse injury model (Marmarou et al., 1994), as seen with the progressive degeneration of white matter in TBI patients (Bendlin et al., 2008), therefore providing better translation.

In addition, it's worth noting that although population-based studies have looked at the dose-response relationship between TBI and its risk towards neurodegenerative outcomes (Chen et al., 2007b; Fleminger et al., 2003b; Gardner et al., 2014b; Plassman et al., 2000; Wang et al., 2012), pre-clinical studies investigating this relationship of the TBI nature and severity on neuropathological outcomes are still scarce (Bramlett et al., 1997; Mouzon et al., 2014). This is crucial for intervention studies to cater specifically for the nature and severity of TBI, thus providing a more accurate and efficient treatment strategies in clinical studies.

Therefore, this study aimed to determine the neuropathological changes especially those associated with early signs of neurodegeneration at one year post diffuse traumatic brain injury of varied severity, in hopes to elucidate the link between TBI and neurodegenerative diseases at a molecular level.

2. Results

2.1. Severity of TBI influences NeuN expression

Cytoplasmic neuronal levels were measured using NeuN marker in all regions of the brain and spinal cord investigated in this study, to determine TBI-related neuronal damage. There were no significant changes in NeuN observed in the striatum ($F_{3,21}=1.4$, $p=0.271$) (Figure 1B) and substantia nigra ($F_{3,20}=0.144$, $p=0.932$) (Figure 1C) with only a trend towards significance in the hippocampus ($F_{3,21}=2.809$, $p=0.065$) (Figure 1A) when compared against groups at 12 months post TBI. However, significant injury effect on NeuN expression was uncovered in the motor cortex ($F_{3,16}=31.07$, $p<0.0001$) (Figure 1D) and all spinal cord regions; cervical ($F_{3,15}=13.14$, $p=0.0002$) (Figure 1E), middle thoracic ($F_{3,15}=16.22$, $p<0.0001$) (Figure 1F) and lumbar region ($F_{3,16}=4.072$, $p=0.025$) (Figure 1G). Post hoc analysis revealed that mild TBI, both single and repetitive nature, had significant elevated levels of NeuN in the motor cortex; mTBI (1.25 ±0.07 vs 0.63 ±0.10, $p<0.0001$ against shams and vs 0.48 ±0.03, $p<0.0001$ against msTBI) and rmTBI (1.12 ±0.04 vs 0.63 ±0.10, $p=0.0005$ against shams and vs 0.48 ±0.03, $p<0.0001$ against msTBI) at 12 months post TBI. Similar elevated levels of NeuN in the

mTBI and rmTBI group were also found in the cervical spinal region; mTBI (2.25 ±0.25 vs 1.50 ±0.12, p=0.035 against shams and vs 1.22 ±0.03, p=0.006 against msTBI) and rmTBI (2.62 ±0.18 vs 1.50 ±0.12, p=0.002 against shams and vs 1.22 ±0.03, p=0.0004 against msTBI) as well as in the middle thoracic spinal region; mTBI (1.77 ±0.15 vs 1.13 ±0.04, p=0.018 against shams and vs 0.91 ±0.12, p=0.001 against msTBI) and rmTBI (1.99 ±0.14 vs 1.13 ±0.04, p=0.002 against shams and vs 0.91 ±0.12, p=0.0001 against msTBI). However, in the lumbar spinal region, only the rmTBI showed elevated levels of NeuN when compared against shams (1.17 ±0.21 vs 0.52 ±0.11, p=0.024 against shams) at 12 months post injury.

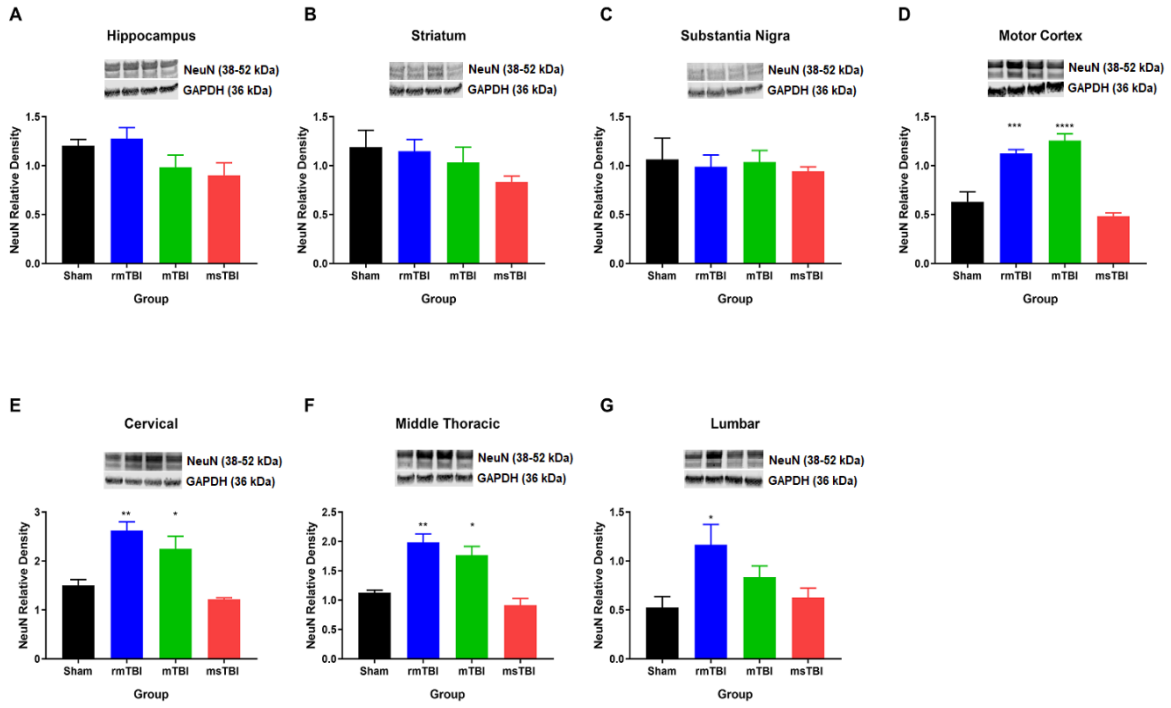

Figure 1. Cytoplasmic neuronal levels were measured by NeuN marker in the A) hippocampus, B) striatum C) substantia nigra, D) motor cortex, E) cervical, F) middle thoracic and G) lumbar of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7), ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 compared to shams. Representative images of the western blots were extracted from Image Studio Lite.

2.2. Synaptic and neuronal structural changes only visible in the motor cortex of mild TBI

Synaptic and neuronal structural damage post injury was assessed using a variety of markers; Synaptophysin for assessing synaptic integrity, NF-L (neurofilament light chain) for assessing neurofilament structure and axonal stability and MBP (myelin basic protein) for assessing neuronal myelination stability.

Synaptophysin expression was unaltered in all brain regions (Figure 2 A-D); hippocampus ($F_{3,21}=0.217$, $p=0.884$), striatum ($F_{3,21}=0.316$, $p=0.814$) and substantia nigra ($F_{3,20}=0.45$, $p=0.72$) at 12 months post injury, with only a trend towards significant change seen in the motor cortex ($F_{3,16}=2.548$, $p=0.092$) (Figure 2D).

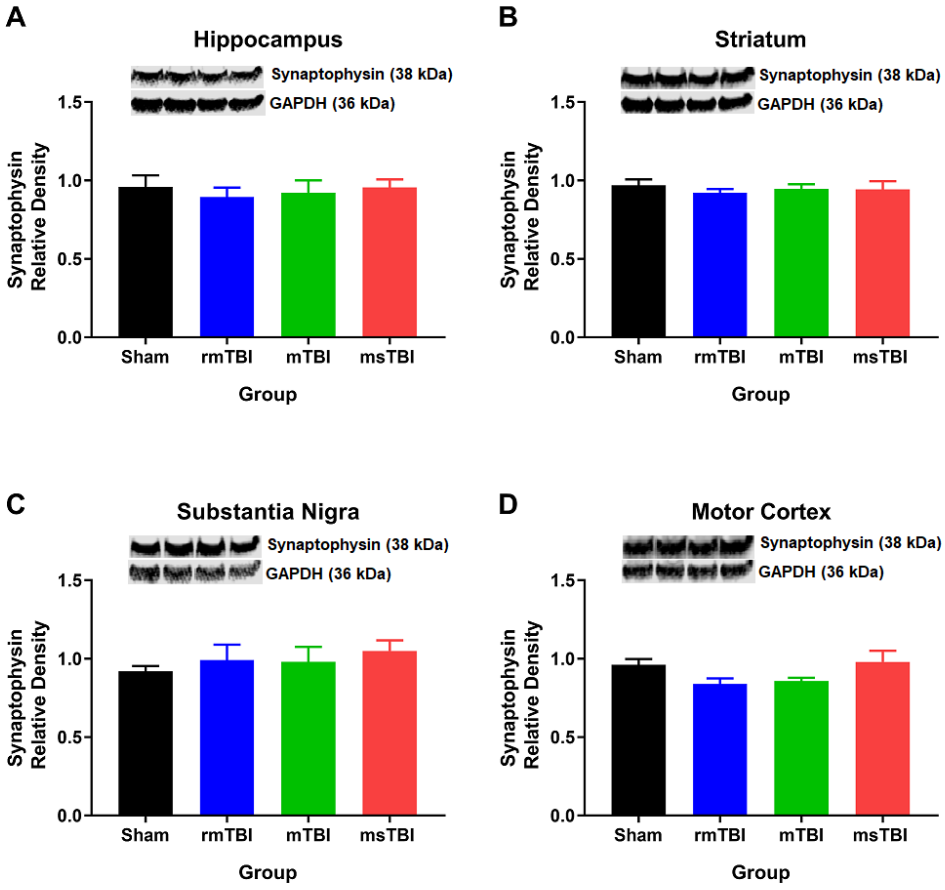

Figure 2. Synaptic changes were measured by synaptophysin in the A) hippocampus, B) striatum, C) substantia nigra and D) motor cortex of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

As for neuronal structure assessed through neurofilament changes (NF-L), there was a significant injury effect observed in the motor cortex region at 12 months post injury ($F_{3,16}=3.288$, $p=0.048$) (Figure 3D). However, post hoc analysis showed no significant changes between injury groups and sham or among the injury groups as well, with only a trend towards a decrease in NF-L in the msTBI when compared against the rmTBI (0.883 ±0.075 vs 1.15 ±0.046, $p=0.061$). Other brain regions showed no significant effect in NF-L at 12 months post injury; hippocampus ($F_{3,21}=1.271$, $p=0.310$) (Figure 3A), striatum ($F_{3,21}=1.336$, $p=0.290$) (Figure 3B), with only a trend towards significance in the substantia nigra ($F_{3,19}=2.485$, $p=0.092$) (Figure 3C).

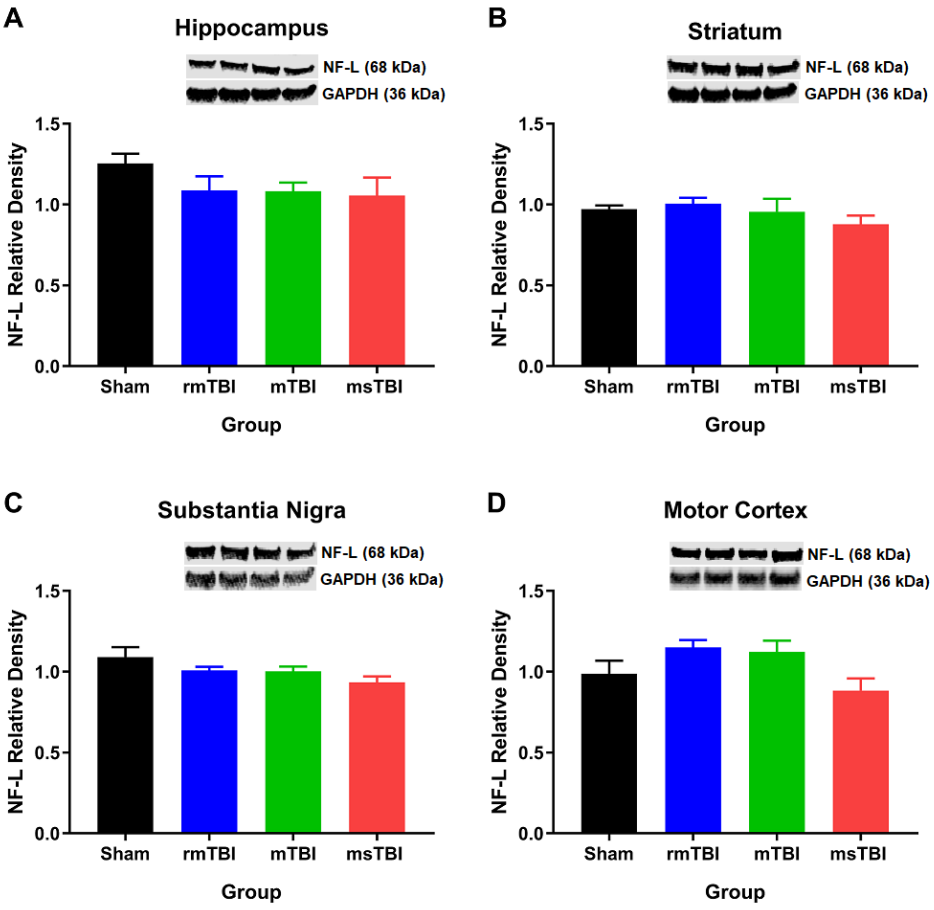

Figure 3. Neuronal integrity was measured by neurofilament light chain (NF-L) in the A) hippocampus, B) striatum, C) substantia nigra and D) motor cortex of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

Myelin integrity assessed through myelin basic protein (MBP), once again only revealed a significant effect in the motor cortex at 12 months post injury ($F_{3,16}=32.45$, $p<0.0001$) where the rmTBI and mTBI animals had MBP levels that were significantly elevated when compared against sham and msTBI (Figure 4D); rmTBI (2.354 ±0.162 vs 0.825 ±0.121, $p<0.0001$ against shams and vs 0.871 ±0.13, $p<0.0001$ against msTBI) and mTBI (2.211 ±0.165 vs 0.825 ±0.121, $p<0.0001$ against shams and vs 0.871 ±0.13, $p<0.0001$ against msTBI). There were no significant MBP alterations seen in the hippocampus ($F_{3,21}=1.212$, $p=0.33$) (Figure 4A), striatum ($F_{3,21}=0.965$, $p=0.428$) (Figure 4B) and substantia nigra ($F_{3,20}=0.870$, $p=0.473$) (Figure 4C) at 12 months post injury.

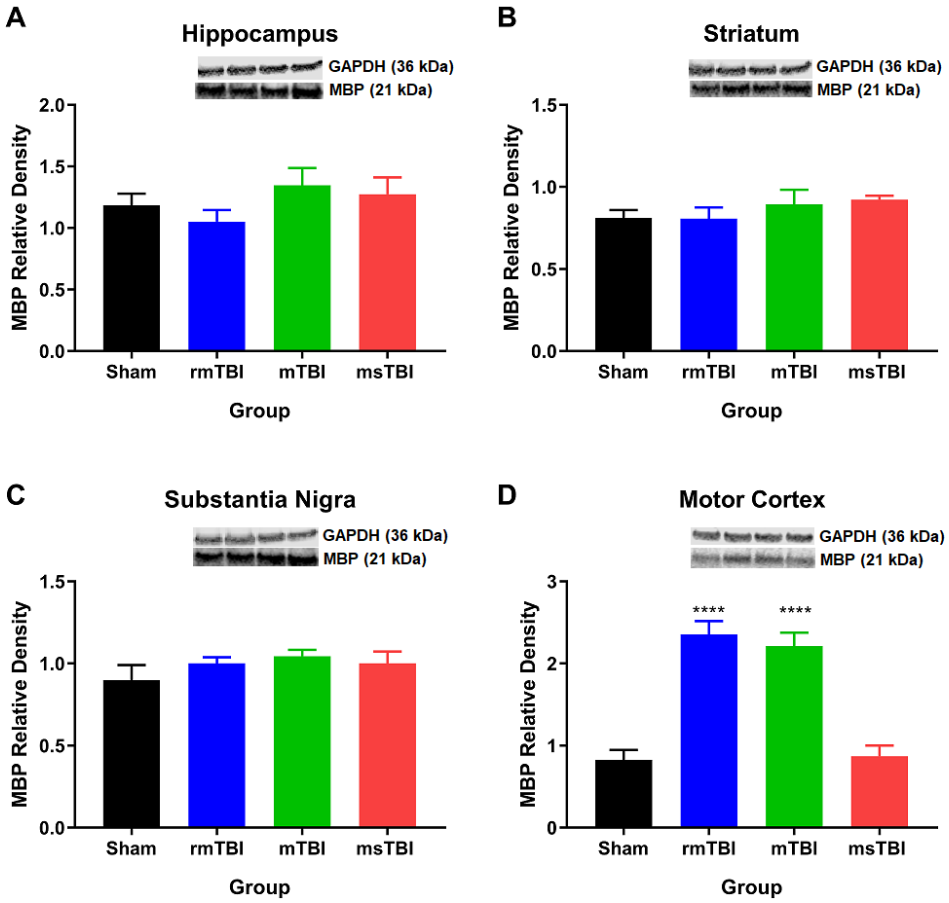

Figure 4. Myelination was measured by myelin basic protein (MBP) in the A) hippocampus, B) striatum, C) substantia nigra and D) motor cortex of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

2.3. Neurodegenerative marker changes

Early changes in proteins association with neurodegenerative diseases were assessed at 12 months post injury in the brain regions relevant for each disease. Tau markers associated with AD (total tau, phosphorylated tau 231 and oligomeric tau) were assessed in the prefrontal cortex and hippocampus, whilst PD was evaluated with levels of alpha-synuclein in the striatum and substantia nigra. Hallmark markers (TDP-43 and phosphorylated TDP-43) as well as an indirect measure of oxidative stress marker (superoxide dismutase-1, SOD-1) were used to investigate ALS pathology in the motor cortex and spinal cord regions post TBI.

2.3.1. Lack of tauopathy after TBI

There was no indication of tauopathy in the prefrontal cortex or in the hippocampus at 12 months post injury. Total tau levels showed no changes in the prefrontal cortex ($F_{3,21}=1.223$, $p=0.326$) (Figure 5A) and in the hippocampus ($F_{3,21}=0.733$, $p=0.544$) (Figure 5E) post injury. Similarly, phosphorylated tau 231 also displayed no changes in the prefrontal cortex ($F_{3,21}=0.144$, $p=0.932$) (Figure 5B) and in the hippocampus ($F_{3,21}=1.95$, $p=0.153$) (Figure 5F). Thus, when evaluating the ratio of phosphorylated tau 231 with the total tau, as expected, no significant differences were found in the prefrontal cortex ($F_{3,21}=1.301$, $p=0.300$) (Figure 5C) or in the hippocampus ($F_{3,21}=1.392$, $p=0.273$) (Figure 5G). Oligomeric tau analysis also revealed insignificant tau pathology in the prefrontal cortex ($F_{3,21}=0.054$, $p=0.983$) (Figure 5D) and in the hippocampus ($F_{3,21}=0.468$, $p=0.708$) (Figure 5H) at 12 months post injury.

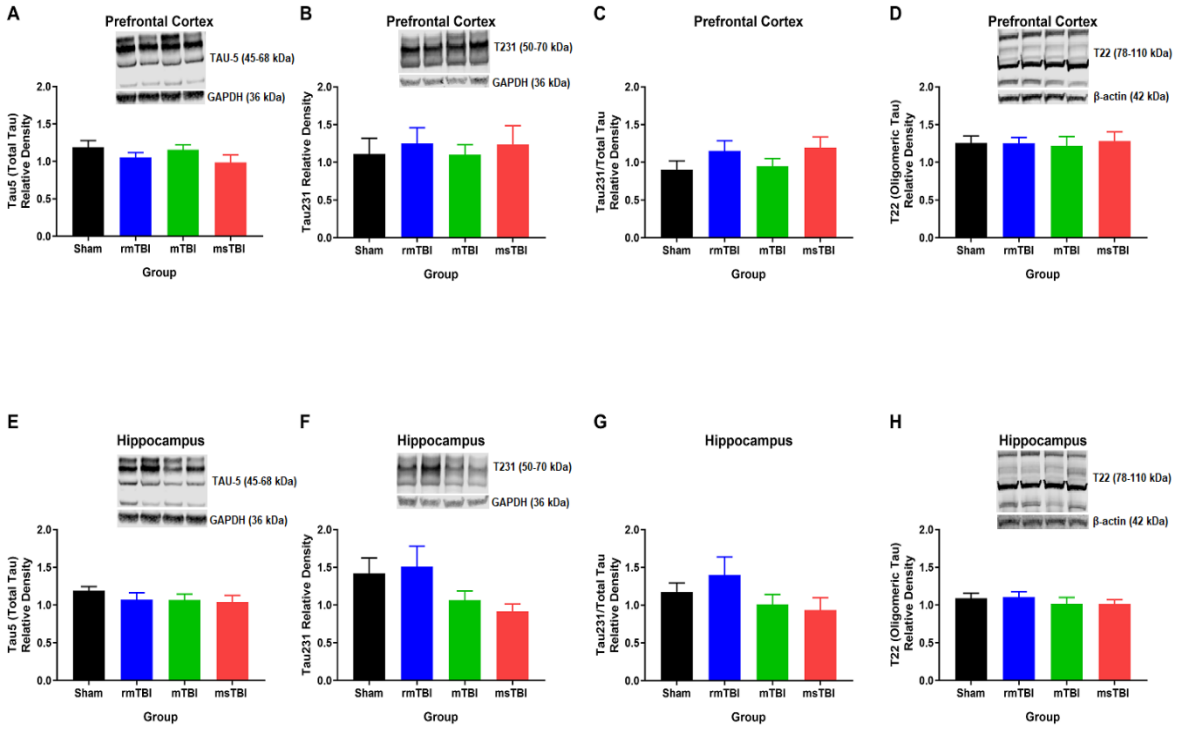

Figure 5. Tauopathy was measured by total tau (TAU-5), tau phosphorylation (Tau231), ratio of phosphorylated tau and oligomeric tau levels (Tau22) in the A-D) prefrontal cortex and E-H) hippocampus of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for TAU-5 and Tau231 analysis while beta-actin was used as a housekeeper protein for Tau22 analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

2.3.2. Alpha-synuclein levels unchanged post injury

Our analysis indicated that TBI, regardless of severity did not affect alpha-synuclein levels in the striatum (F3,21=0.315, p=0.815) (Figure 6A) or in the substantia nigra (F3,20=0.440, p=0.727) (Figure 6B) at 12 months post injury.

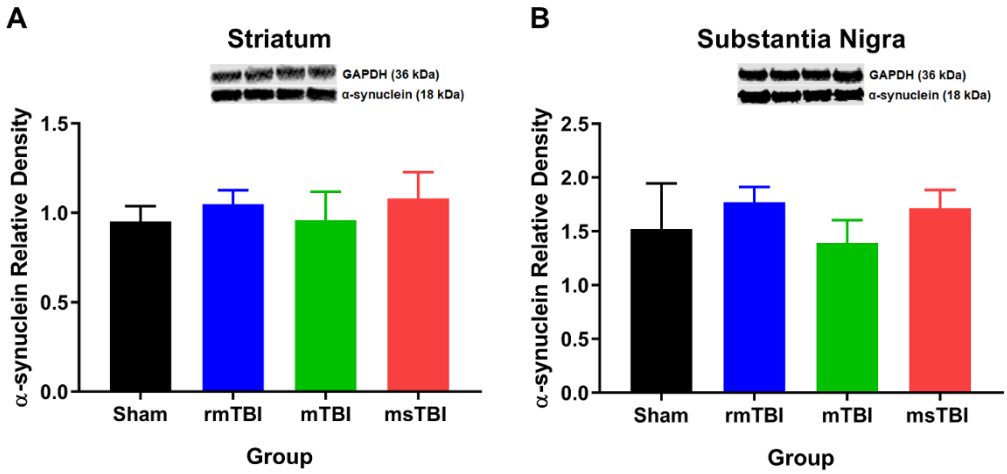

Figure 6. Alpha-synuclein was measured in the A) striatum and B) substantia nigra of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

2.3.3. TDP-43 and phosphorylated TDP-43 changes were seen in motor cortex and spinal cord regions of mild TBI animals

Significant changes in TDP-43 levels were found in the cervical spinal region (F3,16=8.085, p=0.002) (Figure 7A) and in the motor cortex (F3,16=4.698, p=0.016) (Figure 7J) at 12 months post injury. Further analysis in the cervical spinal region showed significantly elevated levels of TDP-43 in the rmTBI (2.245 ±0.222 vs 0.921 ±0.062, p=0.005) and mTBI (1.877 ±0.382 vs 0.921 ±0.062, p=0.046) when compared to shams, with the former also found significantly elevated against msTBI (2.245 ±0.222 vs 0.97 ±0.134, p=0.007). However, in the motor cortex, elevation in TDP-43 levels in the rmTBI (1.748 ±0.162 vs 1.117 ±0.092, p=0.041) and mTBI (1.773 ±0.191 vs 1.117 ±0.092, p=0.032) were only significant against msTBI at 12 months post injury. TDP-43 levels were not different between groups in the middle thoracic spinal

region (F3,16=1.285, p=0.314) (Figure 7D) and lumbar spinal region (F3,16=0.702, p=0.565) (Figure 7G) post injury.

On the other hand, significant changes in phosphorylated TDP-43 (pTDP-43) levels were observed in the cervical spinal region (F3,16=13.85, p=0.0001) (Figure 7B) and in the lumbar spinal region (F3,15=9.369, p=0.001) (Figure 7H) post injury instead. Phosphorylated TDP-43 was found significantly decreased in the cervical spinal region of the rmTBI group (1.035 ±0.027 vs 1.796 ±0.172, p=0.0009) when compared against the cervical spinal region of the sham animals. These pTDP-43 levels of the rmTBI animals were also found to be significantly lower than the cervical spinal region of the msTBI group (1.035 ±0.027 vs 1.679 ±0.091, p=0.004). Similarly, pTDP-43 levels in the cervical spinal region of the mTBI animals were also reduced when compared against shams animals (1.022 ±0.103 vs 1.796 ±0.172, p=0.0008) as well as when compared against msTBI animals (1.022 ±0.103 vs 1.679 ±0.091, p=0.004). Interestingly, the lumbar spinal region also showed a significant decrease in phosphorylated TDP-43 levels in the rmTBI (0.723 ±0.067 vs 1.038 ±0.041, p=0.003) and mTBI (0.718 ±0.057 vs 1.038 ±0.041, p=0.002) when compared against shams at 12 months post injury. Neither the middle thoracic spinal region (F3,15=0.33, p=0.804) (Figure 7E) nor the motor cortex (F3,16=1.978, p=0.158) (Figure 7K) showed any changes in the phosphorylated TDP-43 marker chronically post injury.

To determine if the phosphorylated TDP-43 levels were changing in relation to the total TDP-43 levels, a ratio of these markers were analysed. Significant changes in the phosphorylated TDP-43 to TDP-43 ratio were noted in the cervical spinal region (F3,16=17.19, p<0.0001) (Figure 7C) and in the motor cortex (F3,16=9.676, p=0.0007) (Figure 7L) at 12 months post injury. Post hoc analysis of the cervical spinal region revealed a significant decrease in this ratio seen in the rmTBI group (0.476 ±0.039 vs 1.959 ±0.16, p=0.0002) when compared against shams as well as when compared against the cervical spinal region of the msTBI animals (0.476 ±0.039 vs 1.855 ±0.248, p=0.0004). This ratio was also found reduced in the cervical spinal region of mTBI animals (0.687 ±0.223 vs 1.959 ±0.16, p=0.0009) when compared against

shams and msTBI (0.687 ±0.223 vs 1.855 ±0.248, p=0.002). Similar post hoc results were also seen in the motor cortex of the rmTBI group which showed a lower ratio when compared against shams (0.697 ±0.04 vs 1.246 ±0.162, p=0.009) and msTBI (0.697 ±0.04 vs 1.305 ±0.108, p=0.004), with mTBI also showing decreased motor cortex ratio levels when compared against shams (0.733 ±0.064 vs 1.246 ±0.162, p=0.015) as well as msTBI (0.733 ±0.064 vs 1.305 ±0.108, p=0.007). Despite the lumbar spinal region showing changes in phosphorylated TDP-43, there was insignificant difference when taken in relation to total TDP-43 levels ($F_{3,16}=1.764$, p=0.195) (Figure 7I). The middle thoracic spinal region also showed no changes in this phosphorylated TDP-43 to TDP-43 ratio ($F_{3,15}=1.335$, p=0.300) (Figure 7F).

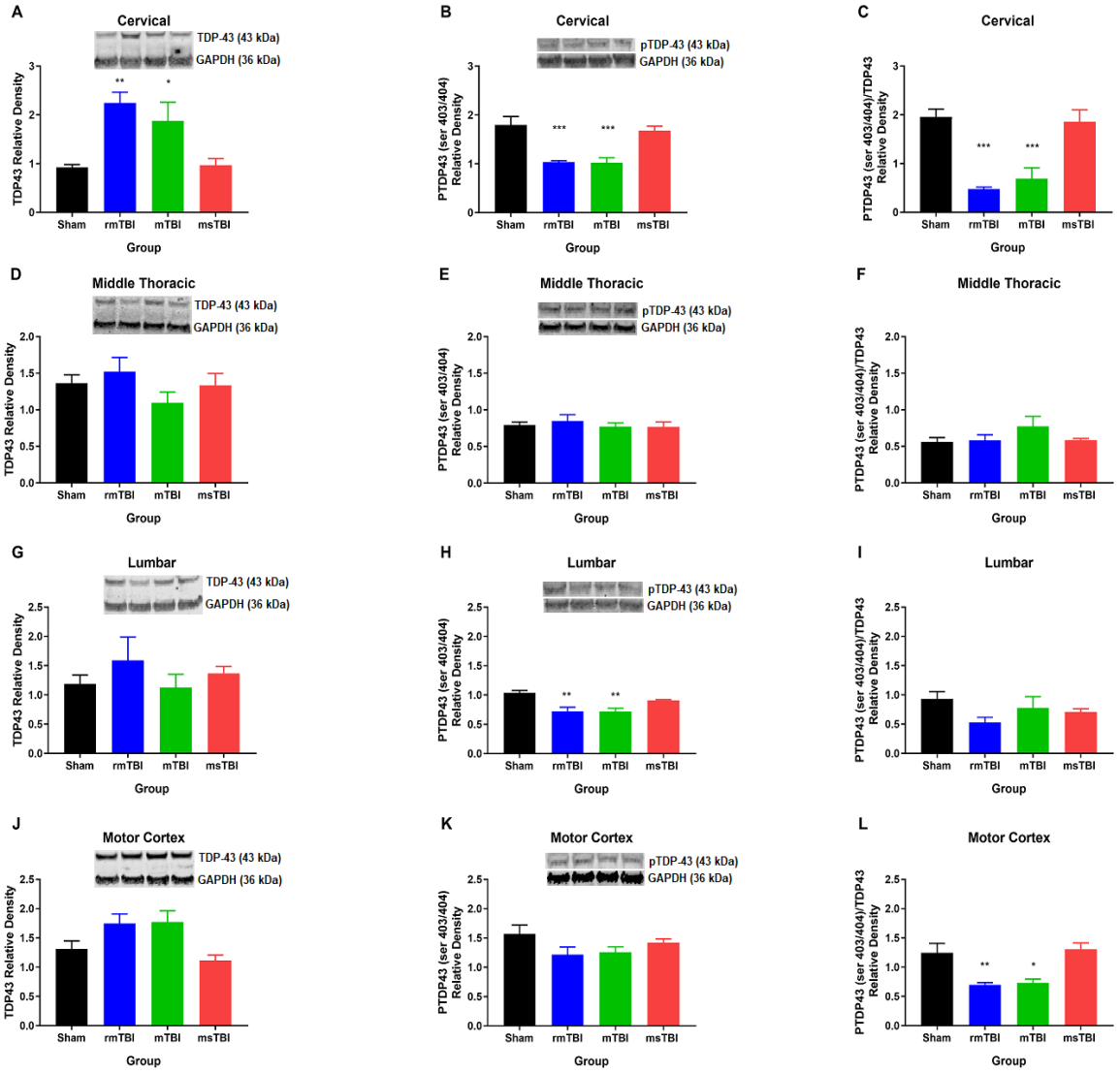

Figure 7. Markers of ALS (TDP-43, pTDP-43 and phosphorylated ratio) were measured in the A-C) cervical, D-F) middle thoracic, G-I) lumbar and J-L) motor cortex of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7), **p<0.01, *p<0.05 compared to shams. Representative images of the western blots were extracted from Image Studio Lite.

An oxidative stress marker (SOD-1) was measured as an indirect assessment of reactive oxygen species which may influence TDP-43 and phosphorylated TDP-43 levels in the motor cortex and spinal cord regions post injury. Despite the changes in the ALS hallmark markers, oxidative stress was not evident in the motor cortex ($F_{3,16}=0.936$, $p=0.446$) (Figure 8D) and in any of the spinal cord regions; cervical spinal region ($F_{3,16}=0.573$, $p=0.641$) (Figure 8A), middle

thoracic spinal region (F3,16=1.981, p=0.158) (Figure 8B) and lumbar spinal region (F3,16=2.1, p=0.140) (Figure 8C) at 12 months post injury.

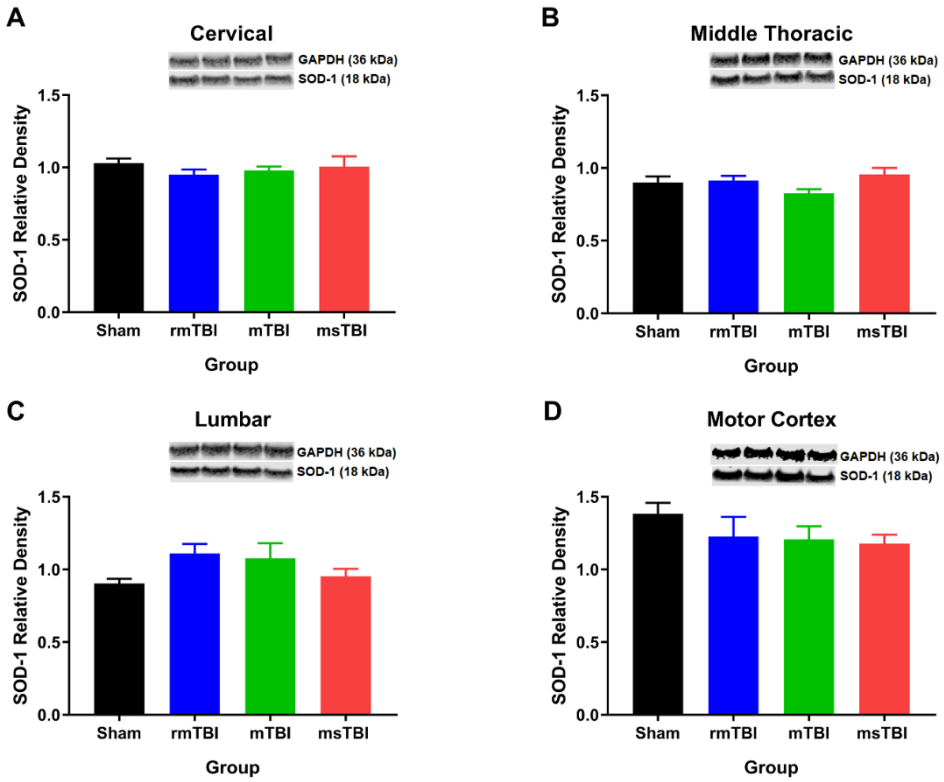

Figure 8. Oxidative stress was measured by superoxide dismutase-1 (SOD1) in the A) cervical, B) middle thoracic, C) lumbar and D) motor cortex of all animal groups at 12 months post injury. GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean± SEM. (n=5-7). Representative images of the western blots were extracted from Image Studio Lite.

3. Discussion

This study set out to investigate the neuropathological link between TBI and neurodegenerative diseases by examining accumulation of abnormal proteins at 12 months following TBI of differing severity. Our results suggest that there may be a link between TBI and ALS due to the subtle cytoplasmic neuropathological changes, particularly in the dysregulation of TDP-43 and phosphorylated TDP-43 proteins, in the motor cortex and spinal cord regions of mild single and mild repetitive TBI. However, this study failed to find pathological markers associated with AD and PD at this time-point post-injury.

ALS is the most known form of motor neurone disease that results in progressive degeneration of both upper (motor cortex and corticospinal tract) and lower (spinal cord) motor neurons leading to muscle atrophy over time (Perry et al., 2010). Thus, one of the key pathological features of ALS is the gradual loss of motor neurons (total number and functionality) in the central nervous system (Perry et al., 2010), attributed to neuronal pathological changes such as the inclusion, overexpression, cytoplasmic mis-localisation and hyperphosphorylation of the TDP-43 protein, creating a toxic environment within neurons (Coan and Mitchell, 2015; Saberi et al., 2015). Briefly, nuclear TDP-43 protein regulation is important for cellular and protein replication and autoregulation, respectively, while low-levels of cytoplasmic TDP-43 protein is mainly found during the shuttling of nuclear RNA/mRNA into the cytoplasm (Barmada et al., 2010; Saberi et al., 2015). Nevertheless, when this tightly regulated protein undergoes upregulation, abnormal cleavage, hyperphosphorylation and high rates of mis-localization into the cytoplasm (Barmada et al., 2010; Johnson et al., 2011b; Lu et al., 2016; Sun et al., 2018), which environmental stressors such as the shearing of axons in TBI may initiate (Johnson et al., 2011b), it causes neurotoxicity and eventual degeneration of neurons. Although abnormal TDP-43 protein and its dysregulation can be found in neurons of any brain regions; in fact it have been implicated in Alzheimer's disease brain pathology as well (McAleese et al., 2017), this TDP-43 pathology is more commonly found in motor neurons, therefore establishing it (TDP-43 and phosphorylated TDP-43) as markers of ALS (Dickson et

al., 2007). Barmada *et al* (2010) discovered that mis-localisation of TDP-43 proteins into the cytoplasmic region of neurons are the main cause for neuronal toxicity and eventual degeneration in ALS which may be due to the TDP-43 loss of function within the nuclear region for DNA replication or the sequestration of mRNA transcripts and inability of mRNA translation cause by the cytoplasmic TDP-43 binding (Barmada et al., 2010). In contrast, another study suggests oxidative stress within the motor neurons; increases in reactive oxygen species and decreases in antioxidant such as SOD-1 (Strong et al., 2005), may be a key contributor to ALS pathology. However, the major finding from this study coincides with the study by Barmada *et al* (2010), in that cytoplasmic TDP-43 were found increased in the cervical spinal region but no changes in SOD-1 were observed post mild TBI, thus providing possible implications towards ALS development at chronic stages post TBI.

Since both the single and repetitive form of mild TBI showed similar elevations of cytoplasmic TDP-43 in the cervical spinal region at 12 months post injury when compared to shams, a dose response relationship may not be established, more so with the lack of this pathology in the moderate to severe TBI group. Past literature on studies involving contact sport athletes supports our results regarding the link between TBI and motor-related outcomes with some studies predicting a high risk factor of motor neurone disease in athletes even after sustaining mild TBI (Belli and Vanacore, 2005; Chio et al., 2005; Lehman et al., 2012; Manley et al., 2017; Peters et al., 2013; Sundman et al., 2014a). Furthermore, similar to our results, moderate to severe TBI patients rarely report long term motor impairments or motor-related pathology (Thomsen et al., 2015) with some suggesting that injury to motor cortex may not result in motor dysfunction but injury induced oxidative damage down the spinal cord have been found to correlate with motor deficits post moderate to severe TBI (Evans et al., 2015). Regardless, in our study, only mild TBI seem to cause motor related cervical pathology, with no evidence of motor cortex or spinal cord pathology in the moderate to severe TBI group, thus, suggesting that TBI severity effect may not affect motor-related outcomes or its associated pathology post injury. Besides that, some studies have reported bulbar onset of ALS pathology

post TBI (Brettschneider et al., 2014; Nair et al., 2010) and therefore could potentially be a region for future studies to investigate especially in regards to moderate to severe TBI.

Since this study only found TDP-43 elevations in the cytoplasm of the cervical spinal region post injury, we believe that there may be spinal cord regional differences in ALS pathology, as supported by Brettschneider *et al* (2014) whose study suggest ALS pathology were confined mainly within the cervical and lumbar spinal region in their patient cohort and indicated these regions as focal points in ALS pathology which will project to other areas of the spinal cord and cortical region as the disease progress (Brettschneider et al., 2014). Moreover, some studies have suggested the cervical spinal region as the key pathological region for ALS; Nair *et al* (2010) showed higher radial diffusivity in the cervical white matter of ALS patients compared to age matched controls with no changes in any diffuse tensor imaging (DTI) measures in the brainstem (Nair et al., 2010), suggesting ALS pathology stems from the cervical spinal region, while Agosta *et al* (2008) also agrees that imaging the cervical spinal cord may provide a useful diagnostic tool for ALS progression (Agosta et al., 2009). Thus far, this ALS progression have not been well studied in the context of TBI and may halt the knowledge of stressors like TBI as initiator of sporadic ALS pathology beginning in the cervical spinal region. In preclinical TBI studies, ALS disease pathology are mainly seen in the cortex region with Wright *et al* (2017) showing motor neurone disease pathology (increases in cytoplasmic TDP-43 and pTDP43) in the motor cortex but not in the lumbar region post 3 months after FPI (Wright et al., 2017b) and Yang *et al* (2014) observing the mis-localization of TDP-43 from the nucleus to cytoplasm in both CCI injury and blast injury (Yang et al., 2014). However, since these studies did not look at the cervical spinal region, it could be speculated that the ALS pathology witness in their study may have stemmed from earlier pathological changes in the cervical region.

Interestingly, some of the changes seen in the motor cortex and the regions of spinal cord in this study negatively correlates with previous experimental TBI-MND research (Wright et al., 2017b), particularly the increases in total neurons (in the motor cortex, cervical and middle

thoracic spinal region) and myelination (in motor cortex) and the decrease in phosphorylated form of TDP-43 (pTDP-43) (in cervical and lumbar spinal region) seen in our post mild and repetitive mild diffuse TBI at 12 months post injury. In addition the ratio of pTDP-43 to the total TDP-43 were observed to be down-regulated in the motor cortex and cervical spinal region of these animals, suggesting that phosphorylation of TDP-43 protein may not be occurring at 12 months post injury, despite the upregulation of TDP-43 proteins. Phosphorylation of TDP-43 have been recognised as an ALS severity marker (Brettschneider et al., 2014), however, phosphorylation of TDP-43 is mediate by protein kinases such as casein kinases (CKI and II) or cell division cycle 7-related kinase (CDC7) (Yamashita et al., 2016). Thus, a dysregulation of these kinases; CKI inhibition may be upregulated by TBI (Dash et al., 2011), may explain the reduction in pTDP-43 levels in the mild TBI groups. Since molecular analysis was not performed for protein kinases levels, the kinase change in these motor neurons could not be determined, thus serving as a limitation of this study.

As for increases in cytoplasmic neuronal levels in the motor cortex and spinal regions post mild and repetitive TBI, one study suggest that increases in cytoplasmic NeuN levels (as seen in this study) in ALS transgenic pigs is due to interactions with cytoplasmic TDP-43 causing mis-localisation of NeuN, which is predominately found in the nuclear region of neurons, into the cytoplasm, thus preventing RNA splicing to continue in the nuclear region of neurons (neurite growth halted) (Wang et al., 2015). Although this may explain the increases in cytoplasmic NeuN in the cervical region of the mild TBI groups, increases in the motor cortex and middle thoracic requires further exploration to determine the reason for this phenomenon in spite of TDP-43 pathology within these motor neurone regions.

Similar to increases in NeuN marker, the MBP marker (used in measuring neuronal myelination) was also found increased in the motor cortex of the mild TBI groups, which may further support the motor neurone disease pathology and may indicate possible ALS development at 12 months in the upper motor neuron network as well. Ajao *et al* (2012) observed that increases in MBP marker in cortical regions of rats correlated with white matter

morphological changes at 2 months post injury which they believe attributed to the motor deficits seen on the rotarod at this timepoint (Ajao et al., 2012). Although they did not relate this findings to possible ALS pathology at 2 months post injury, given the additional NeuN increases and the pTDP-43/TDP-43 ratio decrease (which have been related to ALS pathology), the MBP increase in the motor cortex of the mild TBI groups may suggest the development of upper motor neurone disease (Agosta et al., 2009) post injury.

On the other hand, given the lack of abnormal protein aggregation, regardless of TBI severity, in other brain regions studied such as the striatum, substantia nigra, hippocampus and prefrontal cortex, we suggest that without other exogenous factors, TBI alone may not be sufficient to precipitate later neurodegeneration. Alternatively, the time-point chosen may be too early to detect these changes, given that it equates to late middle age, while tau pathology and alpha-synuclein aggregates are typically seen post mortem-ly in AD/CTE (Castellani and Perry, 2019) and PD patients (Goldman et al., 2012), respectively, years after TBI. However, in preclinical studies, these neurodegenerative pathologies are usually evident acutely (within weeks) post TBI in rodents with its disappearance as the animal ages. For example, Hawkins *et al* (2013), detected oligomeric and phosphorylated tau proteins up to 2 weeks post FPI (Hawkins et al., 2013) with a couple of other studies suggesting earlier detection (less than 7 days for most types of TBI, even of mild severity) (Arun et al., 2015; Gabbita et al., 2005; Liu et al., 2011; Lv et al., 2014a; Shultz et al., 2015), while Acosta *et al* (2015) found alpha-synuclein increases in the substantia nigra at 60 days post CCI injury (Acosta et al., 2015b). Nevertheless, since rarely do these studies investigate these pathologies chronically post experimental TBI, with Mouzon *et al* (2014) and Mannix *et al* (2013) showing no tau pathology at 6 and 12 months post mild TBI (Mannix et al., 2013; Mouzon et al., 2014) and Uryu *et al* (2003) exhibiting recovery of alpha-synuclein pathology at 16 weeks post CCI injury (Uryu et al., 2003), supporting our findings, the possibility of re-emergence of these pathologies at further timepoints (more than 12 months) may still be questionable given the clinical evidence.

In conclusion, this study suggests that at 12 months post injury there may only be evidence of motor-related neurodegeneration such as ALS, which may affect both upper and lower motor neurons, post mild diffuse TBI, regardless of severity. This provides a significant insight to the increase risk of neurodegeneration post experimental TBI, mimicking the clinical evidence. Future studies should investigate the neuropathology at later timepoints (18 months) to ensure that the motor-related neuropathology seen in this study at 12 months in the motor cortex and spinal cord regions of the mild TBI animals, may progress further into widespread motor neuronal loss leading to muscle atrophy in these animals. This study shows that the motor cortex and spinal cord regions are most sensitive towards mild injuries and should be focused more in future studies.

4. Experimental Procedure:

4.1 Animals

Male Sprague-Dawley rats (10-12 weeks) were used under the approval of the University of Adelaide Animal Ethics Committee (M-2015-243A) and (M-2015-187). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery (n=3), repetitive sham surgery (3 incisions at 5 day intervals) (n=3), a single mild diffuse TBI, mTBI (n=6), repetitive mild diffuse TBI, rmTBI (3 mild diffuse injuries at 5 day intervals) (n=7), or moderate to severe diffuse TBI, msTBI (n=7). Following behavioural testing (as described in chapter 3 and chapter 4 previously), animals (12 month cohort only) were saline perfused at 12 months post injury and the brains and spinal cords were collected for molecular analysis.

4.2 Injury Model

The Marmarou impact-acceleration model (Marmarou et al., 1994) was utilized, as it has been extensively validated as a model of diffuse injury (Xiong et al., 2013b). Animal weights ranged from 420-480g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. Animals in the sham, repetitive sham,

mild diffuse TBI and repetitive mild diffuse TBI groups were maintained on 2% isoflurane via nose cone throughout, while animals in the moderate/severe diffuse TBI group were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout (Marmarou et al., 1994; McColl et al., 2018). A midline incision on the scalp was made to facilitate the placement of a metal disc centrally between lambda and bregma on the skull. Animals in the sham and repetitive sham groups receive the incision only, with repetitive sham animals receiving the incision three times at 5 days intervals between each incision.

Animals in the repetitive mild diffuse TBI and mild diffuse TBI group were removed from the nose cone and strapped onto a foam, with injury induced by releasing a 450g weight from a height of 0.75 metres down a clear tube onto the centre of the metal helmet; mild diffuse TBI animals receive this procedure only once, while repetitive mild diffuse TBI animals receive this injury three times at 5 days intervals between each injury (Table 1). Conversely, animals in the moderate to severe diffuse TBI group were transiently taken off ventilation after incision, strapped onto a foam, with injury induced by releasing a 450g weight from a height of 2 metres (Table 1). Contact was observed to ensure a single, direct impact without a rebound hit in all animals. Only animals in the moderate/severe diffuse TBI group were then subjected to hypoxic conditions (2L/min nitrogen; 0.2L/min oxygen) for 10 minutes, to replicate the clinical effects seen following this injury model without ventilation, as this hypoxic condition is known to exacerbate the severity of the injury (Hellewell et al., 2010; Ishige et al., 1987a). Hypoxia alone had similar levels of cytoskeletal structure and neuroinflammation as shams under normoxic ventilation as reported by Hellewell *et al.* previously (Hellewell et al., 2010). Saline treatment (5mL of 0.9% (w/v) saline solution) was administered subcutaneously to prevent dehydration (Eakin et al., 2015a) in the moderate/severe diffuse TBI group after wound closure and if there was continuous weight loss post injury.

Wound closure was performed with surgical staples. Successful induction of moderate/severe TBI was assessed 24 hours later by rotarod scores of below 100, weight reduction of 5-10% and clinical signs (paresis and hunched posture). Animals in the moderate/severe TBI group

that did not meet the above criteria were excluded from the study. Shams, repetitive shams, mild diffuse TBI and repetitive mild diffuse TBI animals assessed at the same timepoint (24 hours) exhibited none of the clinical signs and had rotarod scores of more than 100s.

Table 1. Injury model and specifications

Injury Type	***Weight of metal***	***Height of drop***	***Days of injury***	***Mechanically ventilated***	***Hypoxia Treatment***	***Saline Treatment***
Repetitive Mild TBI	450g	0.75 m	3 days (at 5 day intervals)	No	No	No
Mild TBI	450g	0.75 m	1 day	No	No	No
Moderate to Severe TBI	450g	2.00 m	1 day	Yes	Yes	Yes

4.3 Tissue Collection and Processing

Animals were transcardially perfused with 0.9% saline and the brain and spinal cord were removed. The brain was further dissected into the prefrontal cortex, motor cortex, striatum, substantia nigra and hippocampus (n=5-7 per group) while the spinal cord was further dissected into cervical, thoracic and lumbar regions (n=5-7 per group). All tissue samples were snap-frozen in liquid nitrogen before being stored at -80°C.

The samples were taken out and homogenised in freshly prepared RIPA lysis buffer (150mM sodium chloride, 50mM Tris-hydrochloride acid of pH 7.5-8, 1% of NP-40 IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% of sodium dodecyl sulfate (SDS) and distilled water) with 1X cOmplete^{TM} EDTA-free protease inhibitor cocktail (Sigma). After homogenisation, each sample underwent 3 bursts of 10 seconds duration under a sonicator probe with a cooling period between each burst. Then the samples were centrifuged for 30 minutes at 14000 rpm and 4°C, before the supernatant were collected. Protein concentration was estimated with Pierce BCA Protein Assay Kit (ThermoScientific) with the absorbance read at 540nm. All supernatant were stored at -80°C until further usage.

4.4 Western Blot

Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels (Life Technologies) with 30ug of protein loaded per well. Gels were run at 150V for 1 hour except for oligomeric tau that was run at 100V for 1hour 45 minutes. After the run, blots were transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes x 5 minutes), stained with Ponceau S red solution (Fluka Analytical) (5 minutes) for protein visualisation, and washed with distilled water until sufficient removal of the Ponceau stain had been achieved.

Membranes were then incubated for 5 minutes with the 1X iBind solution before proceeding with the final step of simultaneous incubation with primary and secondary antibodies in 1X iBind solution for 2.5 hours using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations for specific tissue regions as specified in Table 2. Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. The blots were imaged using an Odyssey CLx Infrared Imaging System (model 9140) (LI-COR, Inc.) set at auto resolution for optimum visualisation. Semi-quantitative analysis of band signals were performed using Image Studio Lite version 5.2. Normalization of blot runs were performed using a single control sample (Rapoport et al.) across blots of the same protein of interest. Thus, relative density of the samples was calculated based on the adjusted density for each blot, as below:

$$\text{Adjusted density} = \frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$$

$$\text{Relative density} = \frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$$

Table 2. Molecular markers

Molecular Markers	Company	Dilution	Molecular Weight (kDa)	Function
Oligomeric Tau, T22	Merck (ABN454)	1:250	78, 110	Markers of neurodegenerative disease
Hyperphosphorylated tau, T231	Abcam (ab151559)	1:500	50-70	
Total tau, TAU5	Merck (577801)	1:500	45-68	
Alpha synuclein	Abcam (ab212184)	1:500	18	
TDP-43	Abcam (ab109535)	1:500	43	
Phosphorylated TDP-43 (Ser403/404)	Cosmo Bio (TIP-PTD-P05)	1:500	43	
Superoxide dismutase (SOD-1)	Abcam (ab13498)	1:1000	18	Oxidative Stress
Myelin basic protein (MBP)	Abcam (ab62631)	1:750	21	Myelin structural damage
Neurofilament Light chain (68kDa) (NFL)	Abcam (ab72997)	1:2000	68	Axon structural damage
Synaptophysin	Abcam (ab32127)	1:4000	38	Synapse structural damage
NeuN	Abcam (ab177487)	1:750	38-52	Total neurons
GAPDH	Abcam (ab83957) Abcam (ab9485)	1:1000	36	Housekeeper protein
B-actin	Sigma-Aldrich (SAB3500350)	1:1000	42	Housekeeper protein

4.5 Statistics

All data was analysed via two-way ANOVA (Analysis of Variance) using IBM SPSS statistics 24 and GraphPad Prism software. P values < 0.05 were considered statistically significant. Shams and repetitive shams were combined together as a single sham group, as they did not differ with statistically significance in any molecular analysis.

Discussion, Future Directions And Conclusion

Traumatic brain injury (TBI) is a major and common debilitating disorder of the 21st century (Khoury and Benavides, 2018). Although the advancement of science, medicine and technology has increased survival post-TBI, sadly, despite initial recovery, a percentage of these survivors go on to suffer persistent deficits (Dikmen et al., 1995; Vincent et al., 2014b). Furthermore, increasing evidence suggests that a history of TBI increases the risk for the later development of neurodegenerative disease. Population and clinical studies have shown a dose dependent relationship between TBI and neurodegenerative diseases, whereby mild TBI (either single or repetitive) is associated with a wider range of neurodegenerative disease, such as motor neurone disease (Chen et al., 2007a; Raj et al., 2017), Parkinson's disease (Bower et al., 2003; Factor and Weiner, 1991) and, specifically for repetitive mild TBI, chronic traumatic encephalopathy (McKee et al., 2013b), while moderate to severe TBI is more often linked with dementia (Gardner et al., 2014a; Raj et al., 2017; Salib and Hillier, 1997).

Based on the literature review presented in the first chapter of this thesis, it was found that most preclinical studies investigating the relationship between TBI and its long-term functional outcomes, particularly neurodegeneration, may not accurately model clinical findings, which may contribute to why treatment strategies for the prevention of long-term impairments post TBI have been ineffective to date. This is because most preclinical studies were focused on focal injuries (Kokiko-Cochran et al., 2016; Shultz et al., 2012; Tan et al., 2016; Wright et al., 2017a) or mixed injury models (Byrnes et al., 2012; Gao et al., 2017; Laurer et al., 2001; Thomsen et al., 2016; Zhang et al., 2015), with only a couple carried out in a purely diffuse model, such as the weight drop model (Rachmany et al., 2013) or CHIMERA (Chen et al., 2017a; Nolan et al., 2018b). The clinically relevant 'purely' diffuse injury results in a lower GSC score (greater unfavourable outcome post injury) and has a higher prevalence among TBI patients compared to focal TBI, with 72% of moderate-severe TBI patients categorised as sustaining diffuse TBI on admittance (Skandsen et al., 2010).

Additionally, a majority of the research conducted to date focused on timepoints in the acute to early chronic (1 to 6 months) phase post-injury (Chen et al., 2017a; Lynch et al., 2016; Petraglia et al., 2014; Saber et al., 2017) and, therefore, may not account for the aging effect in relation to functional outcomes post injury. A recent review by Wood R.L. (2017) concluded that the literature to date suggest TBI to accelerate age-related cognitive decline and may lead to premature aging, therefore having possible implications towards early dementia development post injury (Wood, 2017). One study from that literature included showed significantly worse cognitive outcomes, in terms of attention, processing speed, working memory and overall executive function, in long time survivors of TBI compared to acutely injured TBI patients, which was irrespective of age at injury (Senathi-Raja et al., 2010). This suggest that time since injury (aging) may play an important role in determining the functional outcomes post injury and therefore preclinical studies should account for this effect, especially if the relationship between TBI and age-associated diseases like dementia is of concern.

Finally, only a handful of studies have tried to determine the effect of TBI severity on long-term functional outcomes post-injury (Gao et al., 2017; Laurer et al., 2001; Mouzon et al., 2014; Petraglia et al., 2014), with only one study thus far that has investigated all three types of TBI severity (single mild TBI, repetitive mild TBI and moderate to severe TBI) within a single study (Thomsen et al., 2017). This is significant as trying to compare severity effects across studies may introduce confounding variables, such as animal species, TBI models and age at induction of injury. Thus, when all these gaps and limitations in the current literature are taken together, the overarching aim of this thesis was to investigate long-term functional and neuropathological outcomes following different severities of TBI in a diffuse axonal injury model, up to 12 months post injury.

As preclinical investigations on outcomes following moderate to severe diffuse TBI are scarce within the literature (Byrnes et al., 2012; Erturk et al., 2016; Thomsen et al., 2017), this thesis first sought to determine the early chronic functional and neuropathological changes up

to 3 months post-injury in a model of moderate/severe 'purely' diffuse TBI. The functional outcomes from this study suggest that neuropsychiatric impairments, mainly a depressive-like phenotype, are the first to develop at 1 month post injury and remained persistent up to 3 months, followed by subtle cognitive changes in flexibility that developed at 3 months post moderate to severe diffuse TBI, which is consistent with the clinical outcomes following an injury of similar severity (Draper et al., 2007; Himanen et al., 2006; Kaup et al., 2017; Palacios et al., 2013), with some preclinical studies observing persistent elevations of immobility at early chronic time points (2-3 months) post moderate to severe diffuse TBI in the weight drop model (Milman et al., 2005) as well as in a focal and more severe CCI model (Taylor et al., 2006). Depression has also been associated with repetitive mild TBI, clinically (Hart et al., 2013; Vargas et al., 2015b) and pre-clinically (Briggs et al., 2016; Petraglia et al., 2014; Shultz et al., 2012; Tan et al., 2016), where the higher number of hits (severity of repetitive TBI) increased the likelihood of depressive-like behaviour on the forced swim test; 95g weight drop (Briggs et al., 2016) or 5 mild FPI (Shultz et al., 2012) displayed longer immobility time than 75g weight drop or 3 mild FPI, respectively. However, whether moderate to severe TBI was more associated with a higher risk and persistence of depression post injury compared to repetitive mild TBI, was only investigated in chapter 3, which showed no depressive-like phenotype at 12 months regardless of TBI severity.

On the other hand, cognitive flexibility impairment witness at 3 months post injury in the moderate to severe TBI in this chapter 2 was found to persist up to 12 months (chapter 3 and chapter 4) in this severity group, thus suggesting persistent executive function impairment beginning as early as 3 months post moderate to severe TBI but not in milder forms, supporting clinical findings (Maillard-Wermelinger et al., 2009; Muscara et al., 2008a). Since the impairment in cognitive flexibility at 3 months was in spite of learning and memory deficits, together with the depressive-like phenotype witness, the study believes that the diffuse moderate to severe TBI may have created a greater damage in the prefrontal cortex (PFC) region, as executive function and emotion are PFC-dependent behaviour (Bizon et al., 2012; Rao et al.,

2010) while learning and memory are more hippocampal-dependent (Conrad et al., 1996; Epp et al., 2013). Thus, the neuropathology to support these behavioural impairments was further investigated.

While it is still unclear what brain mechanisms may account for these early chronic functional changes, previous research has suggested that neuroinflammation may be driving these impairments (Cherry et al., 2016; Erturk et al., 2016; Johnson et al., 2013; Kokiko-Cochran et al., 2016; Webster et al., 2015). However, in our study, we failed to show any elevation in either the total number of microglia or astrocytes at 3 months post injury, when the majority of the functional outcomes were displayed. Thus, the study concluded that neuroinflammation and oxidative stress present in the prefrontal cortex at 1 month may initiate an as yet unexplored downstream pathology (for example: synaptic and receptor dysfunction) that may lead to the deficits observed at 3 months. Interestingly, this neuropathology was only evident in the prefrontal cortex, and not in the hippocampus. The prefrontal cortex is critical for neuropsychiatric and executive function (Chou et al., 2016b; Nolan et al., 2018b; Stuss, 2011) while the hippocampus is more associated with learning and memory (Bird and Burgess, 2008). Given the findings (behavioural and neuropathology) from this study, the PFC may be suggested as being particularly vulnerable to moderate/severe diffuse axonal injury sub-acutely (1 month) post injury. It is imperative to note, however, that our study only examined the total number of microglia, and therefore confirming the differences in the levels between the resting state and reactive state of the microglia may provide a better understanding of the neuroinflammation link chronically post TBI (Cherry et al., 2016; Johnson et al., 2013). Additionally, due to the complexity of neuroinflammatory pathways, future studies should also determine the changes in the levels of chemokines and cytokines, which play a significant role in the pathway between neuroinflammation and neuronal damage (Kokiko-Cochran et al., 2016; Webster et al., 2015). This will allow for a more in-depth analysis of long-term changes in the neuroinflammatory response following TBI.

Given that changes in depressive-like behaviour and cognitive flexibility were present up to 3 months post-injury in our model of moderate/severe TBI, we next wanted to characterise whether these changes were still present at a much more chronic time point (12 months post-injury) and whether the initial severity of the injury impacted functional outcomes. We had hypothesised that functional deficits would worsen over time and would show a dose-dependent effect, with more severe injury associated with greater long-term impairment, consistent with the clinical literature. Surprisingly, at 12 months post injury, there were no major behavioural deficits noted, irrespective of TBI severity, with only subtle cognitive deficits and anxiety-like behaviour observed in the moderate to severe TBI animals. These results seem to indicate that there is behavioural recovery in rodents at this time point, which corresponds to "middle age" in humans. It may be that deficits re-emerge with age, so a longer time-course post-injury is needed to fully explore this. This would be consistent with the clinical literature, since, in humans, the risk of neurodegenerative disease in those with a history of TBI increases with age, with most individuals not showing symptoms until above 55 years old (Breteler et al., 1995; Chen et al., 2007a; Gardner et al., 2014a; Gardner et al., 2015b; McKee et al., 2013b; Mehta et al., 1999; Nordstrom and Nordstrom, 2018; Salib and Hillier, 1997; Tolppanen et al., 2017).

Since neurodegenerative disease risk also increases with the severity of the TBI, whereby individuals with a history of moderate to severe TBI may present with symptoms earlier (higher risk) than those with a history of mild TBI (Fann et al., 2018; Gardner et al., 2015b; Kaup et al., 2017; Nordstrom et al., 2014; Tolppanen et al., 2017), it may be that the subtle behavioural impairments observed at 12 months in the moderate to severe TBI animals would worsen over time, setting the stage for the development of neurodegenerative disease. Typically, however, if this were the case, we would anticipate that we would be able to detect the beginnings of neuropathological change in relevant brain areas. As demonstrated in the final chapter, this was not the case, with the majority of markers assessed showing no alteration. It is important to note, however, that this was a fairly preliminary analysis of the neuropathology, using only Western blot to investigate total protein levels of markers of interest. A more in-

depth investigation, expanding the marker panel to include measures of neuroinflammation and cellular stress or probing alterations in connectivity using neuroimaging, may reveal the beginning of more subtle changes, but was outside the scope of the current thesis.

While the cognitive changes seen following moderate/severe TBI at 12 months were similar to those noted at 3 months in our earlier study, the neuropsychiatric changes were different, with no increase in depressive-like behaviour seen at 12 months post-injury. This may be due to several factors. First, there were significant differences in body weight of the two cohorts, with rodents at 12 months weighing approximately 900g (~900g) and rodents in the 3 month group significantly smaller at around 500g. Given that the forced swim test is dependent on the rodent fitting into a cylinder with a specific diameter, it may be that the larger size in the 12 month animals affected the immobility time measured. Thus, this test may not be an appropriate measure of depressive-like behaviour in older animals, and future studies, utilising alternate tasks, will be needed to fully assess the relationship between TBI and chronic depression.

Additionally, our rodent cohort (i.e. differences in breeding colonies) may be a confounding variable between the two studies. Despite the fact that Sprague-Dawley rats were used for both studies, the breeding population differed between cohorts of animals utilised in the studies and these two cohorts may have differed in their response to the diffuse injury. Previous work from our lab (i.e. Stephanie Plummer's unpublished 2018 thesis) showed that rodents from the two breeding populations displayed differences in gross morphology of the skull, as well as the severity of the clinical signs, such as paresis and mortality rate within 24 hours post injury. While the starting weight of the animals used in the 12 month study was rectified in order to result in a similar severity of clinical signs following injury as that seen in the 3 month study (at least for the moderate to severe TBI group, as clinical signs in the model of mTBI may be unnoticeable), it may be that differences still existed which could affect the depth or extent of the injury. It is also possible that the sample size of the study is

underestimated. Given that even in the human population, there is not a 1:1 relationship between TBI and neurodegeneration (i.e. not all individuals who have experienced a TBI will go on to develop neurodegenerative diseases; often less than 10%) (Barnes et al., 2018; Breteler et al., 1995; Nordstrom and Nordstrom, 2018; Raj et al., 2017; Spangenberg et al., 2009), the same probability may also be present in our preclinical model. Therefore, the small sample size of 14 animals per TBI severity may be masking functional impairments seen in animals that are more susceptible towards long term deficits post injury. Thus, future studies should account for this probability and raise the sample size accordingly.

Given that we saw subtle changes in cognitive flexibility at 12-months post-injury in the moderate to severe TBI animals, we next explored these effect more fully by delving deeper into the temporal profile of changes in executive functioning using a touchscreen cognitive chamber to perform the 5-choice serial reaction time task (5CSRTT). Executive function deterioration is commonly reported in TBI clinical studies (Alosco et al., 2017; Azouvi et al., 2004; Esopenko et al., 2017; Kaup et al., 2017; McDonald et al., 2002; Rochat et al., 2013), and changes in executive function are often the first signs/symptoms of cognitive deficits in dementia patients (Clark et al., 2012). The 5CSRT task utilising the touchscreen chamber has previously been used to measure cognitive impairments in models of Alzheimer's disease (Romberg et al., 2013b) and psychiatric disorders (Nithianantharajah et al., 2013), but has not previously been used following TBI.

Since this was the first time that this task had been used both following TBI and in an aged cohort, first optimisation of the task needed to be performed. Previous studies have set the number of months needed for the training phase, established a paradigm of one hour or 100 trials per testing time and have trained animals up to 0.6s of stimulus presentation (Barnes et al., 2012; Romberg et al., 2013b), whereas in this study, due to behaviour timeline constraints and the age of the animals, the training phase was shortened to 25 days, with 30 minutes or 60 trials per testing time and with animals only trained to 2.5 secs of stimulus presentation.

Animals in the 12 month cohort were bigger in size than the other timepoint cohorts (1 and 6 months) and thus their agility and motivation in the study was less (Amancio-Belmont et al., 2017) compared to the 1 month and 6 month animal cohorts. However, to avoid potential confounders, all testing parameters were kept constant for all animal cohorts, which resulted in a higher number of animal drop outs (unable to achieve criterion) in the 12 month cohort; 1 animal in the 1 month cohort, 6 animals in the 6 month cohort and 10 animals in the 12 month cohort, were excluded from the study. Moreover, due to the shortened training phase, this study's initial objective in conducting the 5CPT (5 choice continuous performance task), which provides a better insight into vigilance and inhibition impairments (Mar et al., 2013a; Young et al., 2009), was halted, as the animals failed to learn the go/no-go task within the short timeframe, and the 5CSRT task was completed instead.

Using the 5CSRT task, the strongest effect seen was that of age, where motivation, attention and reaction time all decreased with age. This was most apparent in the moderate-severe TBI group, where a significant but subtle cognitive flexibility impairment was also observed at 12 months post-injury, consistent with our earlier findings in the Barnes maze at this time point. Moreover, the study found injury severity as a factor that influenced the age-related cognitive changes where different severities affected different domains of executive function over time; single mild TBI in attention, motivation and reaction time, repetitive mild TBI in reaction time only while moderate to severe TBI in attention, reaction time and impulsivity. This is also consistent with the clinical literature, which demonstrates that the severity of TBI affects the age-related decline in executive function; with moderate to severe TBI resulting in greater executive dysfunction in flexibility, speed and attention compared to milder TBI even after 10 years post injury, but that even mild TBI is able to show this deficits post injury (Maillard-Wermelinger et al., 2009; Muscara et al., 2008a). Clinical studies in athletes with repeated concussion also support the decrease in reaction speed seen in this study with Pederson *et al* (2014) suggesting that visual-motor speed decreased significantly after the second concussion in ice-hockey players (Pedersen et al., 2014b). To note, this study saw a

contradicting impulsivity finding, where the literature suggest impulsivity to increase with time since injury (Shaver et al., 2019b) and injury severity (Vonder Haar et al., 2017), however, the moderate to severe TBI in this study showed a decrease in impulsivity over time which was not evident in other severity groups. Dalley *et al* (2002) and Pattij and Vanderschuren (2008) suggest that impulsivity in rodents can decrease if the 5HT system is dysregulated such as increases in noradrenaline (Dalley et al., 2002; Pattij and Vanderschuren, 2008). Thus, the moderate to severe diffuse TBI may have impaired this system (Kobori et al., 2006) causing a gradual decrease in impulsivity over time. In addition, impulsivity decreases as a function of age where studies show adult animals to be less impulsive than those in adolescence (Doremus-Fitzwater et al., 2012; Sasamori et al., 2018) concurrent with population studies (Chamorro et al., 2012; Romer, 2010). This suggest moderate to severe TBI may have increased the vulnerability of the rodents towards age-related decline in impulsivity.

Setting age aside, injury severity itself may have a huge burden on executive function post injury; moderate and severe CCI injury animals displayed significant executive function impairments across all facets tested (attention, impulsivity and motivation) up to 14 weeks post injury while mild CCI only showed persistent impulse control (Vonder Haar et al., 2016). Although this study only showed significant cognitive flexibility impairment against sham animals at 12 months post injury, this study believes that diffuse TBI, which creates widespread axonal damage, may take a longer time to show impairments in other executive function domains compared to CCI (as early as 2 weeks post injury), which precisely targets the PFC area responsible for executive function (Vonder Haar et al., 2016). Taken together, this supports the moderate to severe TBI cognitive outcome in this study of diffuse TBI at 12 months especially in terms of cognitive flexibility impairments compared to other groups over time, and suggest that moderate to severe diffuse TBI may have the strongest impact on the axons of the frontal lobe area which governs these cognitive processes (Bizon et al., 2012) compared to milder diffuse TBI at 12 months post injury. Thus, alterations in executive dysfunction over

time may show a dose dependent relationship, with more severe injury associated with steeper patterns of decline.

Additionally, our results indicate that with more effective optimisation of the 5CSRT or 5CPT (longer training periods to ensure animals have appropriately learned the task), the touchscreen cognitive chamber may become a very useful and informative cognitive tool for assessing the subtle temporal profile of cognitive change post-TBI. A more complete characterisation of these changes is a critical first step in predicting which individuals may be at increased risk for the later development of dementia.

As discussed above, we were interested in whether there were neuropathological changes present at 12 months, specifically an accumulation of proteins associated with neurodegenerative diseases, despite the lack of significant functional impairments at this time point. Such a finding would have supported the idea that there were changes occurring cellularly at this time point that could drive the later re-emergence of neurodegenerative-like functional deficits. Despite this, in our study, which only used Western blot to measure levels of the most common neurodegeneration-associated proteins in brain areas relevant to each disease, we found that TBI was not associated with accumulation of either hyperphosphorylated tau (ptau), or α-synuclein, which are associated with dementia and Parkinson's disease, respectively. This is inconsistent with previous clinical literature, which suggests that there are increases in both tauopathy (Johnson et al., 2012a; Yang et al., 2017) and in alpha-synuclein inclusions (Mondello et al., 2013; Shahaduzzaman et al., 2013) in TBI patients over time. Preclinical studies have also found similar findings in both ptau (Cheng et al., 2014; Hawkins et al., 2013; Huber et al., 2013; McKee et al., 2015) and alpha synuclein (Acosta et al., 2015a; Impellizzeri et al., 2016; Uryu et al., 2003) within the weeks after injury. The lack of ptau findings in this study could be attributed to a lack of neuropathology-causing severity; Yang *et al* (2017) suggested that only severe and extremely severe TBI may result in phosphorylation of tau (Yang et al., 2017), thus the severities of TBI used in this study may not be sufficient to cause tau

phosphorylation. The lack of alpha-synuclein findings may be attributed to a possible recovery stage at 12 months post injury; Uryu *et al* (2003) suggested that alpha-synuclein levels returned to sham levels by 16 weeks post moderate CCI injury (Uryu et al., 2003), therefore suggesting these levels may still remain at sham levels at 12 months post injury; possibly no implications of TBI towards PD.

Furthermore, no differences in markers of neuronal integrity between groups were noted within the PFC, hippocampus, striatum or SN. However, alterations in TDP-43 were seen within the motor cortex and cervical and lumbar spinal regions of mild TBI animals. This may provide preliminary evidence for a relationship between mild TBI and the early stages of ALS. Interestingly, while both the levels of phosphorylated TDP-43 (a neuropathological hallmark of ALS), as well as the ratio of phosphorylated TDP-43 against total TDP-43, were decreased in these areas, there an increase in total TDP-43 levels. As the study did not investigate kinase protein levels such as casein kinases (CKI and II) or cell division cycle-7 related protein kinase (CDC7), where a down-regulation of these kinases may reduce the phosphorylation of TDP-43 (Yamashita et al., 2016), it could be suggested that mild TBI may have affected the translation and regulation of these kinases (Dash et al., 2011), thus causing the decrease in pTDP-43 levels. While a decrease in pTDP-43 levels contradicts the levels associated with ALS, dysregulation of CKI may defend neuronal cells from apoptosis (Dash et al., 2011); thus may also explain the lack of severe motor impairments at this timepoint (no neuronal loss). However, since kinase levels were not investigated and motor function was not robustly assessed in the current study (just open field for general locomotion), future studies should incorporated kinase activity analysis and utilise motor function test that evaluates fine motor impairments, to better relate TBI to motor neurone disease.

As the neuropathology at only one timepoint (i.e, 12 months post-injury) was assessed, the possibility of earlier pathology driving the deficits seen at 12 months remains to be investigated. This is based on the results from the 3 month study, described in chapter 2, that

showed significant neuropathology at 1 month, months before cognitive deficits were observed at 3 months, which had seemingly resolved at 3 months instead. Therefore, a similar pattern may be displayed at 12 months as well, with earlier neuropathological change initiating a downstream cascade that was not investigated in this study, such as neurochemical changes, receptor dysfunction or functional connectivity. As discussed earlier, due to the scope of the current thesis, the neuropathological analysis conducted to date is fairly primitive. Thus, a more in-depth investigation, with an expanded marker panel, may be needed to fully understand the cascade and temporal profile of neuropathological change. Additionally, white matter tract or connectivity damage is often associated with a purely diffuse injury, such as in this study, as axonal shearing is a hallmark of weight drop TBI model (Braun et al., 2017; Narayana, 2017; Xiong et al., 2013a). However, in the current study, only molecular analysis was done, which may not paint the full picture of the brain changes seen at chronic time points following TBI. Thus, future studies should include neuroimaging techniques, such as diffuse tensor imaging (DTI) and magnetic resonance imaging (MRI), to investigate white matter connectivity and volume alterations.

Taken together, this thesis provides some evidence of subtle functional impairments, particularly in cognitive and neuropsychiatric function, at both early chronic and later time points following diffuse TBI. These changes appeared to be dependent on the severity of the initial injury, with moderate to severe TBI having the most impact on long-term functionality. These changes may be indicative of increased risk for future neurodegenerative disease with older age, but a large body of work remains to be done to adequately address this question. This is particularly true given that only a single neurodegenerative marker (TDP43) and no markers of neuronal morphology were altered at 12 months post injury, making it difficult to speculate about a conclusive relationship between TBI and future neurodegeneration. Future studies should pursue further timepoints (at least 18 months post-injury), assess a wider range of neurodegenerative markers and expand investigations beyond changes in neurodegenerative disease related proteins and structural markers to investigate variables such as white matter

changes, neuroinflammation and neuronal network integrity (receptors and neurotransmitters). Nevertheless, this thesis provides significant insight into the long-term outcomes post diffuse injury in a preclinical model that is dependent on the nature of the initiating insult. Although subtle, understanding the temporal profile of functional deficits and accompanying neuropathological changes that occur in the months and years following TBI is critical for improving the predictability of neurodegenerative disease risk following TBI.

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APPENDIX

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Research report

Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury

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ABSTRACT

TBI is a significant risk factor for the development of dementia, with the interaction between structural damage from TBI and neuroinflammation potentially driving this relationship. This study investigated the early chronic post-TBI neuroinflammatory response and its relationship to both neurodegenerative pathology and functional impairment up to 3 months post-injury. Sprague-Dawley rats underwent either sham surgery or the Marmarou model of diffuse moderate-severe TBI. At 1-month and 3-months post-injury, a functional battery encompassing motor function, depressive-like behaviour, anxiety and cognition was performed. Western blot and immunohistochemical analysis assessed a range of inflammatory, neurodegenerative and oxidative stress markers. At both 1 and 3-months post injury, depressive-like behaviour was significantly increased in TBI animals, with TBI animals also exhibiting impaired cognitive flexibility at 3 months, although learning and memory remained intact. This was accompanied by a significant decrease in markers of synaptic integrity and astrocytic and microglia number within the pre-frontal cortex at 1-month post-injury, although this resolved by 3-months post-injury. In contrast, minimal pathology was evident within the hippocampus at 1 month, with only a decrease in neurofilament-light seen at 3 months post-injury. Thus, following a moderate-severe diffuse injury, the pre-frontal cortex is most vulnerable to early neuro-structural changes. While these changes are resolved at 3 months post-injury, future studies should investigate whether they re-emerge or progress to other areas, such as the hippocampus, at later time points, which could predispose individuals to the development of dementia.

1. Introduction

Traumatic brain injury (TBI) represents one of the leading causes of mortality and disability worldwide. The Centre of Disease Control and Prevention stated that, in 2010 alone, there were approximately 2.5 million emergency department visits, hospitalisations and deaths due to TBI [1,2]. There is increasing evidence to suggest that neuronal injury is ongoing following a TBI [3–5], and that moderate-severe TBI may lead to progressive neurodegeneration, such as dementia and associated cognitive and behavioural deficits. Population based studies following patients with moderate-severe TBI showed these functional deficits persisting years later, even after motor function recovery [6–8]. An Australian health survey of TBI cases reported an overall decrease in mental health quality and elevated depression levels when compared to a matched non-TBI cohort, even up to 15 years after injury [9].

Indeed, following a focal injury, lesion volume was found to increase nearly 5 fold over one-year post-injury [10], whereas, following a mixed focal/diffuse injury induced by lateral fluid percussion, cortical and hippocampal tissue loss increased significantly from one week to one year post-injury [11]. This is supported by clinical imaging studies, which have shown progressive white matter damage, particularly within the frontal and temporal regions, as well as loss of cortical grey matter, up to a year post-injury [12], in line with reports of progressive reductions in brain volume as assessed up to 14 months post-TBI [13].

The exact mechanisms that drive this ongoing neuronal injury are yet to be fully elucidated, with the development of an aberrant persistent chronic neuroinflammatory response thought to be one key mechanism [14]. Indeed, multiple studies have demonstrated that a neuroinflammatory response may persist following resolution of the acute effects of a TBI, with inflammatory markers present in the brain parenchyma, serum and cerebrospinal fluid of TBI patients at chronic time points (months to years later) [15–18]. In rodents, microglial activation has been demonstrated up to one-year following a focal TBI, with associated progressive lesion expansion, hippocampal degeneration, myelin loss and oxidative stress [10].

Although a number of studies have shown progressive neuronal loss

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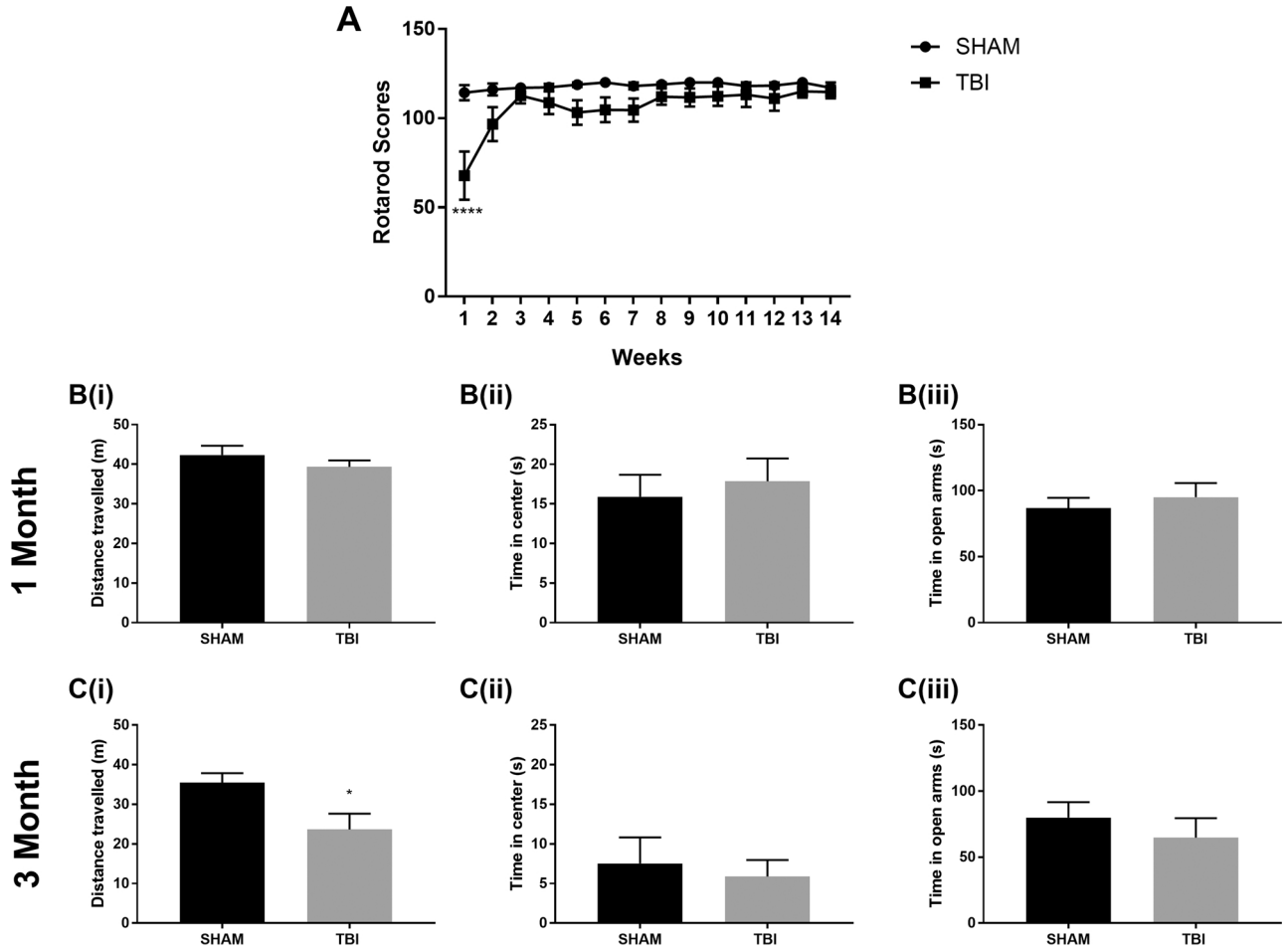

Fig. 1. Functional outcomes measured post injury. A) Motor outcome as measured on the rotarod, weekly for 3 months. Locomotor activity as measured on the open field at B(i)) 1 month and C(i)) 3 months. Anxiety-like behaviour as measured in the open field at B(ii)) 1 month and C(ii)) 3 months and on the elevated plus maze at B(iii)) 1 month and C(iii)) 3 months. Graphs represent the mean ± SEM, (n = 13–19 per group; ****$p < 0.0001$,*$p < 0.05$ compared to shams).

up to one year post-injury, a more detailed examination of the events that occur in the sub-acute and early chronic stages post-TBI that may promote this ongoing neuronal injury have received less attention. Furthermore, these studies have been predominantly conducted utilising focal [10] or mixed focal models [11], rather than a purely diffuse injury. A diffuse injury model would be clinically more relevant as it mimics the hallmarks seen in majority of the human TBI cases (motor vehicle accidents) such as unconsciousness post injury and widespread diffuse axonal injury [19,20]. As such, this study sought to investigate the effects of a moderate-severe diffuse TBI at 1 and 3 months post-injury on synaptic and axonal integrity and neuroinflammation, as well as on functional outcome.

2. Results

2.1. Motor outcome

Motor outcome was assessed weekly up to 3 months (Fig. 1A) on the rotarod. Sham and TBI animals showed no significant differences in their pre-training rotarod scores but a significant injury effect on the scores was seen in the weeks following the injury ($F_{1,19} = 5.146$, $p = 0.035$). TBI animals showed a significantly impaired rotarod scores when compared to shams (67.8 ± 13.47 s vs 114.3 ± 4.23 s in sham animals, $p < 0.0001$) at 24 h post injury (indicated by week 1 on Fig. 1A). However, by the third week (day 15) post-injury, TBI animals had returned to sham levels, (112.7 ± 4.46 s vs 117 ± 2.09 s, $p > 0.9999$) and maintained this for the rest of the testing period.

2.2. Locomotor activity

General locomotor activity was assessed as the distance travelled in the open field test (OFT). At 1 month post-injury (Fig. 1B(i)), TBI animals showed no difference in locomotor activity compared to shams (39.36 ± 1.6 m vs 42.35 ± 2.3 m in shams; $t(32) = 1.089$, $p = 0.2843$), but at 3 months post-injury (Fig. 1C(i)), there was a significant decrease in locomotor activity in the TBI animals when compared to shams (23.6 ± 4.0 m vs 35.5 ± 2.4 m; $t(23) = 2.479$, $p = 0.0209$).

2.3. Anxiety-like behaviour

Anxiety-like behaviour was measured as time spent in centre of OFT and time spent in the open arms of the elevated plus maze (EPM). No significant differences in time spent in centre of OFT and open arms of EPM were seen between the TBI animals and sham animals at 1 month (17.9 ± 2.9 s vs 15.9 ± 2.8 s in shams; $t(32) = 0.485$, $p = 0.6314$) and (95.0 ± 10.8 s vs 86.7 ± 7.9 s in shams; $t(32) = 0.591$, $p = 0.5587$) respectively (Fig. 1B (ii) and (iii)). Similarly, at 3 months post-injury, no significant differences were seen between groups in time spent in centre of OFT (5.9 ± 2.1 s vs 7.5 ± 3.3 s in shams; $t(23) = 0.427$, $p = 0.6735$) (Fig. 1C(ii)) as well as time spent in open arms of EPM (64.8 ± 14.7 s vs 80.0 ± 11.7 s in shams; $t(23) = 0.798$, $p = 0.4329$) (Fig. 1C(iii)).

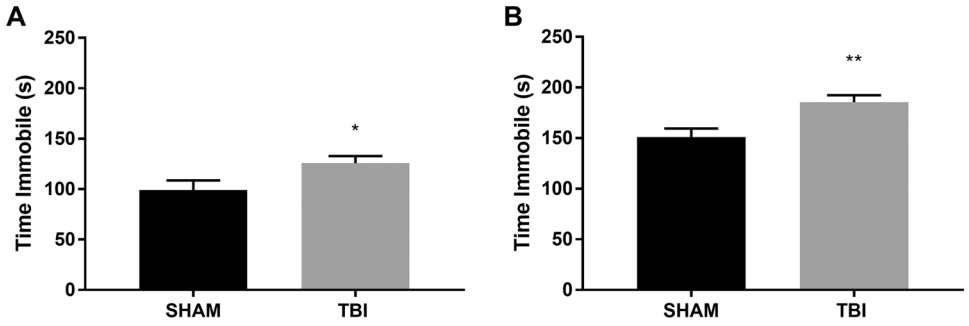

Fig. 2. Depressive-like behaviour as measured in forced swim test at A) 1 month and B) 3 months. Graphs represent the mean ± SEM, (n = 13–19 per group; **p < 0.01, *p < 0.05 compared to shams).

2.4. Depressive- like behaviour

Depressive-like phenotype was assessed based on the immobility time in the forced swim test (FST). TBI animals spent more time immobile than shams at 1 month post-injury (125.8 ± 7 s vs 99.2 ± 9.4 s in shams; $t(32) = 2.32$, p = 0.0269) (Fig. 2A), with this persisting at 3 months post-injury (185.6 ± 6.8 s vs 151.2 ± 8.4 ss in shams; $t(23) = 3.217$, p = 0.0038) (Fig. 2B).

2.5. Cognition

Cognitive outcome was assessed using the Y-Maze for spatial memory and Barnes maze for learning, memory and cognitive flexibility (ability to reprogram previously learned task) (Fig. 3). Y-Maze was performed at 1 month and 3 months post-injury, while the Barnes maze was only performed on the 3 month animals. Spatial working memory in the Y-Maze showed no significant changes in novel preference between the TBI group and the sham control group at any of the time points post-injury; 1 month (0.37 ± 0.03 vs 0.42 ± 0.03 in shams, $t(32) = 1.009$, p = 0.3205), 3 month (0.37 ± 0.04 vs 0.39 ± 0.03 in shams, $t(23) = 0.326$, p = 0.7475) (Fig. 3A–B). On the Barnes Maze, no significant differences were noted in time taken to locate the escape box on any of the training days during the acquisition phase ($F_{1,23} = 0.049$, p = 0.8276)(Fig. 3C). Nor was there any difference in ability to locate the old escape box on the probe day (shams 27.8 ± 12.6 vs TBI 14.0 ± 4.2 s; $t(23) = 1.076$, p = 0.293) (Fig. 3D). In terms of learning the location of the new escape box on probe day, there was a trend of injury effect ($F_{1,23} = 3.979$, p = 0.0581). The sham animals showed greater cognitive flexibility taking a significantly shorter time on Trial 1 compared to TBI animals (63.0 ± 16.3 s vs 24.0 ± 4.6 s in shams, p = 0.014), although both groups had similar times on trial 2 (24.0 ± 7.7 s vs 15.24 ± 3.1 s in shams, p = 0.528) (Fig. 3E).

2.6. Early chronic neuroinflammatory changes in prefrontal cortex (PFC) post-TBI

Levels of inflammation were assessed by counting the number of cells that were immunopositive for GFAP (glial fibrillary acidic protein) (Fig. 4), a structural protein in astrocytes and IBA1 (ionized calcium binding adaptor molecule 1) (Fig. 5), a calcium binding protein seen in microglia within the PFC and hippocampus. At 1 month post injury, GFAP immunopositive staining (GFAP + ve) was decreased within the PFC in TBI animals (135.8 ± 14.39 cells/mm^2) compared to shams (193.4 ± 13.48 cells/mm^2) ($t(7) = 2.92$, p = 0.019) (Fig. 4C). However, the number of GFAP + ve cells in the hippocampus of TBI animals (193.0 ± 22.9 cells/mm^2) and shams (207.5 ± 5.91 cells/mm^2) did not differ significantly between groups ($t(7) = 0.55$, p = 0.601) (Fig. 4D). At 3 months post injury, the number of GFAP + ve cells did

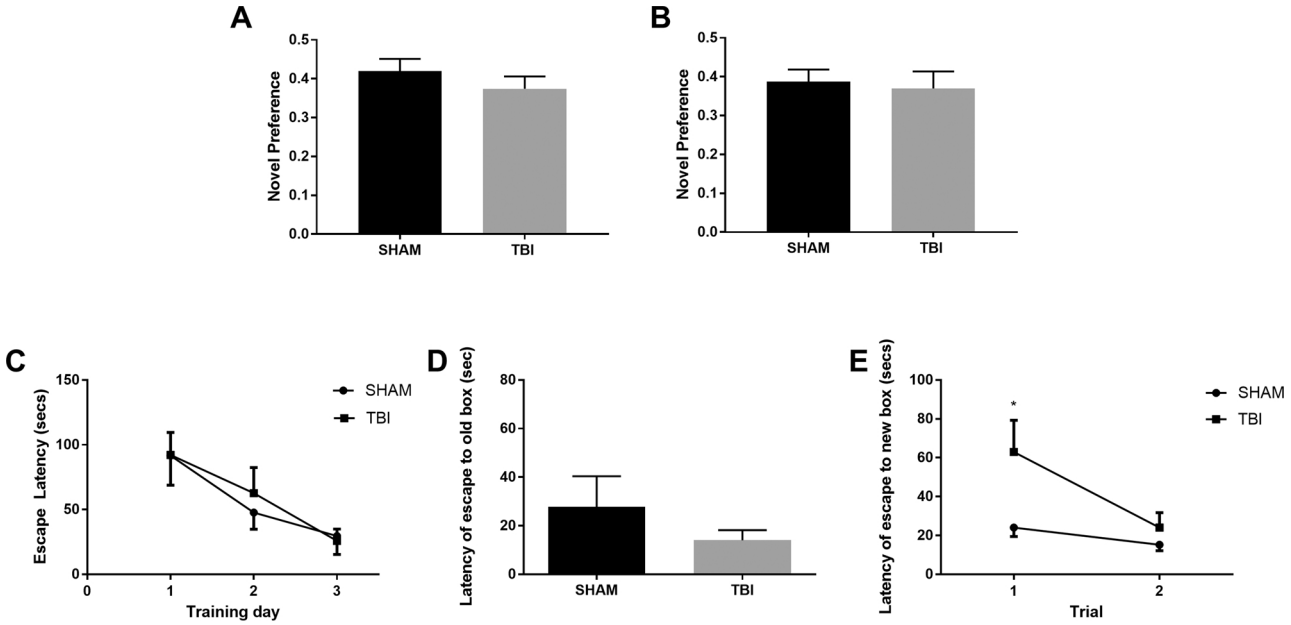

Fig. 3. Cognition assessed through Y-maze for spatial working memory at A) 1 month and B) 3 months post-injury and the Barnes maze at 3 month post-injury (C–E). For the Barnes Maze, C) learning ability in the acquisition phase, D) recollection memory during the probe trial and E) cognitive flexibility on probe day are shown. All graphs show mean ± SEM, (n = 13–19 per group; *p < 0.05 compared to shams).

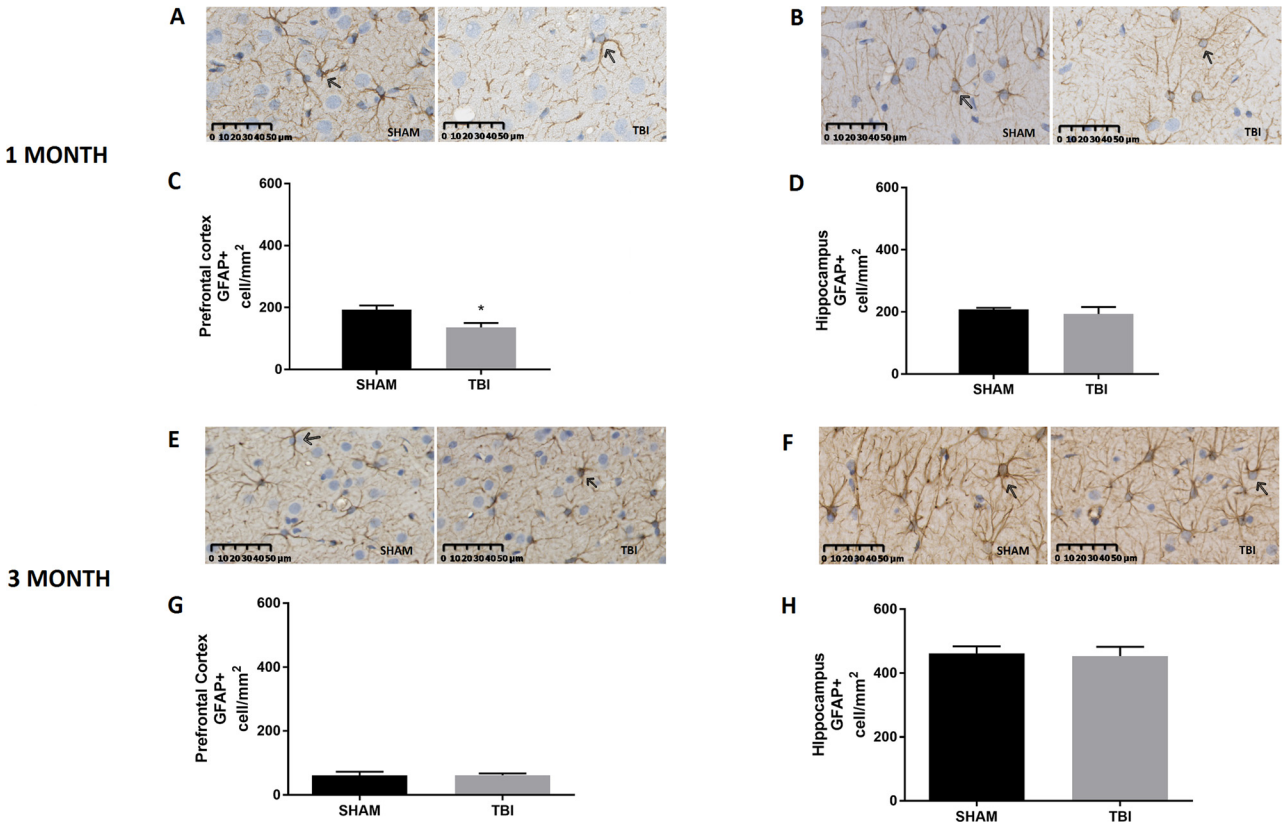

Fig. 4. Representative images of GFAP staining within the A,E) PFC and B,F) hippocampus at A–B) 1 month and E–F) 3 months post-injury, as well as their respective cell counts at C–D) 1 month and G–H) 3 months. Graphs represent the mean ± SEM, (n = 4–5 per group; *p < 0.05 compared to shams).

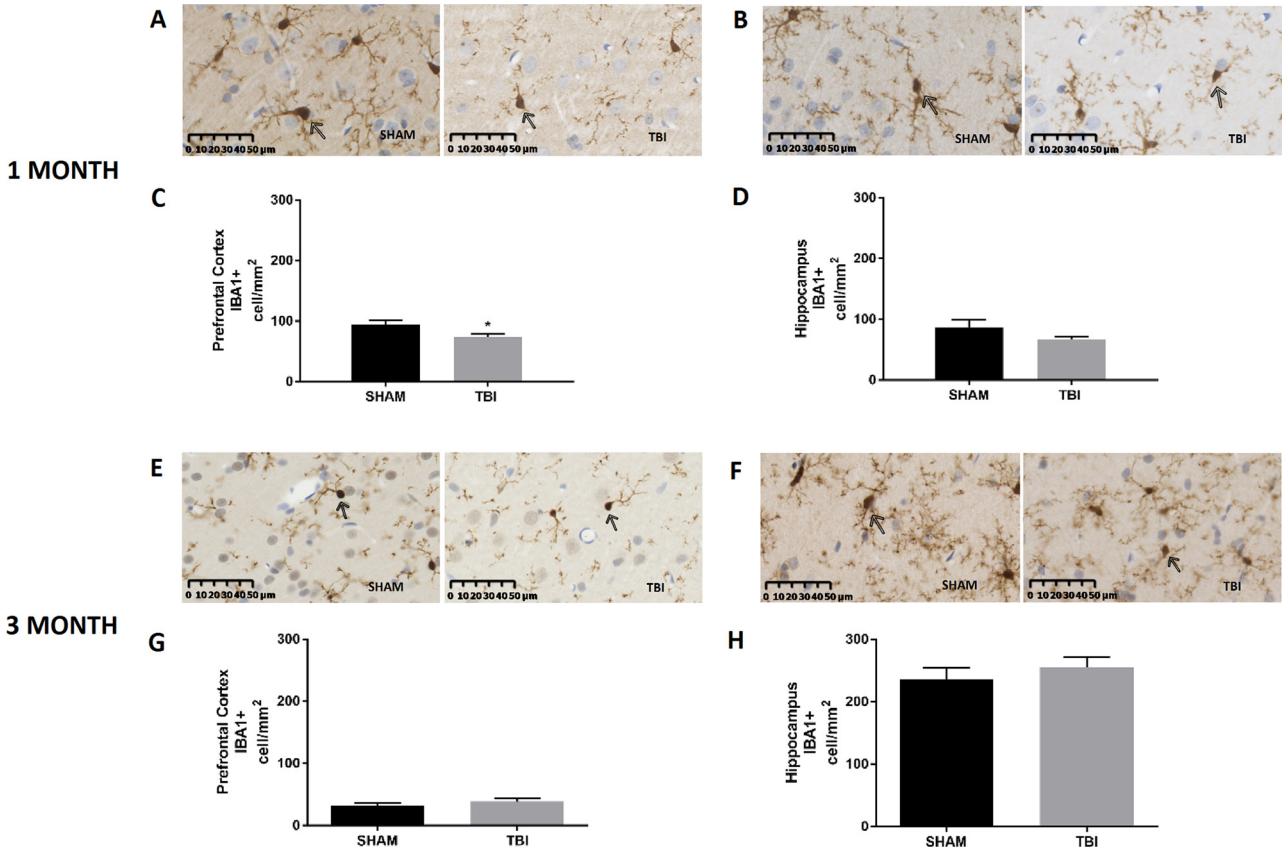

Fig. 5. Representative images of IBA1 staining in the A,E) PFC and B,F) hippocampus at A–B) 1 month and E–F) 3 months, as well as their respective cell counts at C–D) 1 month and G–H) 3 months. Graphs represent the mean ± SEM, (n = 4–5 per group; *p < 0.05 compared to shams).

not significantly differ between the shams and TBI in either the PFC (62.19 ± 5.67 cells/mm^2 vs 61.61 ± 11.58 cells/mm^2 in shams, $t(6) = 0.052$, $p = 0.96$) or the hippocampus (453.5 ± 29.01 cells/mm^2 vs 461.6 ± 22.11 cells/mm^2 in shams, $t(7) = 0.214$, $p = 0.84$) (Fig. 4G–H).

Similarly, the number of IBA1 + ve cells in the PFC of TBI animals (73.84 ± 5.48 cells/mm^2) was significantly decreased compared to shams (94.45 ± 6.70 cells/mm^2) ($t(7) = 2.33$, $p = 0.049$) at 1 month post-injury (Fig. 5C). In contrast, the hippocampus showed no significant differences in IBA1 + ve staining in the TBI animals (66.78 ± 5.03 cells/mm^2) compared to shams (86.99 ± 12.2 cells/mm^2) ($t(7) = 1.54$, $p = 0.176$). By 3 months post-injury, there was no significance difference in IBA1 + ve staining in the PFC (38.54 ± 4.72 cells/mm^2 vs 32.39 ± 3.96 cells/mm^2 in shams, $t(6) = 0.89$, $p = 0.409$) or the hippocampus (254.9 ± 17.28 cells/mm^2 vs 235.9 ± 18.5 cells/mm^2 in shams, $t(7) = 0.75$, $p = 0.478$) between the groups (Fig. 5G–H).

2.7. Evaluation of neuronal and synaptic integrity

Neuronal and synaptic structural damage post injury was assessed using a variety of markers; PSD-95 (postsynaptic density protein 95) and synaptophysin for assessing synaptic integrity, NF-L (neurofilament light chain) and NF-H (neurofilament heavy chain) for assessing neurofilament structure and axonal stability and MBP (myelin basic protein) for assessing neuronal myelination stability. In the PFC, at 1 month post injury, the relative density of PSD-95 and synaptophysin were significantly reduced in the TBI animals compared to shams (0.964 ± 0.214 vs 1.721 ± 0.041, $t(6) = 2.64$, $p = 0.039$ and 2.103 ± 0.469 vs 3.623 ± 0.229, $t(5) = 2.59$, $p = 0.049$, respectively) (Fig. 6). This had resolved by 3 months post-injury, with similar values reported in TBI and sham animals; PSD-95 (1.717 ± 0.322 vs 1.738 ± 0.031 in shams, $t(6) = 0.049$, $p = 0.962$), synaptophysin (1.77 ± 0.268 vs 1.72 ± 0.192 in shams, $t(8) = 0.144$, $p = 0.889$). In comparison, in the hippocampus, there were no significant differences in the relative density of PSD-95 and synaptophysin at either 1 month; PSD-95 (0.474 ± 0.056 vs 0.369 ± 0.037 in shams, $t(8) = 1.57$, $p = 0.154$), synaptophysin (0.786 ± 0.085 vs 0.973 ± 0.107 in shams, $t(6) = 1.363$, $p = 0.222$) or 3 months; PSD-95 (0.239 ± 0.028 vs 0.272 ± 0.024 in shams, $t(7) = 0.877$, $p = 0.41$), synaptophysin (0.519 ± 0.085 vs 0.515 ± 0.123 in shams, $t(8) = 0.022$, $p = 0.983$) post-injury (Fig. 6E–H).

Assessment of axonal integrity with NF-L found no significant differences within the PFC (1.393 ± 0.082 vs 1.405 ± 0.059 in shams, $t(8) = 0.123$, $p = 0.905$) or the hippocampus (1.009 ± 0.076 vs 1.113 ± 0.069 in shams, $t(8) = 1.019$, $p = 0.338$) at 1 month-post-injury; however, a trend towards a decrease in the hippocampus at 3 months post-injury was observed (0.88 ± 0.107 vs 1.184 ± 0.071 in shams; $t(6) = 2.38$, $p = 0.06$) (Fig. 7D). In contrast, a significant increase in levels of NF-H was seen at 1 month post-injury within the PFC (1.398 ± 0.11 vs 0.922 ± 0.138 in shams; $t(8) = 2.70$, $p = 0.027$), which had resolved by 3 months post-injury (0.838 ± 0.148 vs 1.216 ± 0.234 in shams, $t(8) = 1.365$, $p = 0.209$). No changes in NF-H were noted within the hippocampus at 1 month (1.459 ± 0.213 vs 1.5 ± 0.101 in shams, $t(8) = 0.177$, $p = 0.864$) or at 3 months (2.352 ± 0.427 vs 2.642 ± 0.204 in shams, $t(7) = 0.561$, $p = 0.593$) post injury. Integrity of myelin was evaluated with MBP, with a trend towards an increase in the PFC at 1 month post-injury (0.635 ± 0.068 vs 0.404 ± 0.074; $t(7) = 2.304$, $p = 0.055$) which had resolved by 3 months post-injury (1.006 ± 0.018 vs 0.966 ± 0.07 in shams, $t(8) = 0.54$, $p = 0.604$) (Fig. 7I & K). No differences in MBP were seen at 1 month (1.121 ± 0.151 vs 0.915 ± 0.109 in shams, $t(6) = 1.102$, $p = 0.313$) or 3 months (0.494 ± 0.062 vs 0.571 ± 0.103 in shams, $t(7) = 0.595$, $p = 0.571$) post-injury in the hippocampus.

2.8. Oxidative stress

Oxidative stress was assessed by evaluating levels of the antioxidant, SOD-1 (superoxide dismutase 1) (Fig. 8). In the PFC, there was a significant increase in the relative density of SOD-1 at 1 month post-injury (1.082 ± 0.033 vs 0.85 ± 0.032 in shams, $t(6) = 5.074$, $p = 0.002$), which had resolved by 3 months (0.67 ± 0.102 vs 0.602 ± 0.049 in shams, $t(7) = 0.547$, $p = 0.602$) post-injury. In the hippocampus, no changes in SOD-1 were noted at either time-point; 1 month (0.934 ± 0.043 vs 0.881 ± 0.052 in shams, $t(8) = 0.787$, $p = 0.454$), 3 months (0.679 ± 0.056 vs 1.09 ± 0.203 in shams, $t(7) = 1.764$, $p = 0.121$).

3. Discussion

The current study investigated the effect of moderate-severe TBI on chronic changes in axonal and synaptic integrity, neuroinflammation and persistent functional deficits at 1 and 3 months post-injury. It was found that, following TBI, animals showed persistent depressive-like behaviour with increased time spent immobile in the FST at 1 and 3 months post-injury. A decrease in cognitive flexibility on the Barnes Maze was seen at 3 months post-injury, but no impairment was noted in learning and memory during the acquisition phase of the task nor in recognition memory on the Y-Maze (Table 1). Within the PFC, synaptic loss was noted at 1 month post-injury, as indicated by decreased levels of synaptophysin and PSD-95, which corresponded to a concomitant decrease in the number of astrocytes and microglia. Furthermore, other neuronal changes such as increases in NF-H and MBP, were also observed at this early timepoint in the PFC. These changes were resolved by 3 months post-injury. In contrast, within the hippocampus, no changes in the number of inflammatory cells was noted at either time-point nor any effect on synaptic integrity, with the main finding a decrease in relative expression of NF-L at 3 months post-injury.

The most notable functional finding was that TBI led to the development of persistent depressive-like behaviour that had not resolved by 3 months post-injury (Table 1). Although no ongoing motor impairment was noted on the rotarod, with performance at sham level at 3 weeks post-injury, there was a decrease in locomotor activity at 3 months post-injury on the open field. This may relate to lack of motivation to explore the open field [21], but further studies will be needed to confirm this theory. Nonetheless, it appears that the increase in immobility time in the FST reflects a behavioural response, rather than gross motor impairment. This increase in immobility time is thought to be indicative of behavioural despair and, given that it decreases with administration of antidepressants [22], is thought to provide an indicator of depressive-like behaviour. The observations in this study are in line with clinical studies, which have reported the prevalence of depression in TBI patients to be as high as 77% [23], with 30–40% of individuals suffering from major depressive disorder within a year post-injury [24]. In contrast, pre-clinical studies have had mixed results, with reports of no difference in behaviour on the FST at 1 month post moderate controlled cortical impact [25–27] or 6 months post-lateral fluid percussion injury [28]. Conversely, Milman et al and Taylor et al found increased immobility at 2–3 months post-injury utilising a diffuse weight drop model and a more severe CCI model, respectively [29,30]. This suggests that, in order for depressive-like behaviour to be present at sub-acute-chronic time-points post-injury, a wider spread injury may be required, like the diffuse model of injury employed here.

Indeed, within this study, the profile of deficits, in depressive-like behaviour and reduced cognitive flexibility, align with structural changes that were mostly noted within the PFC and not the hippocampus (Table 2). The PFC plays a central role in emotional regulation, with reductions in PFC volume following TBI associated with the development of depressive symptoms post-TBI [24,31,32]. In regards to cognitive flexibility, lesions within the PFC lead to an impairment in the ability to modify a response in relation to new information of a learned

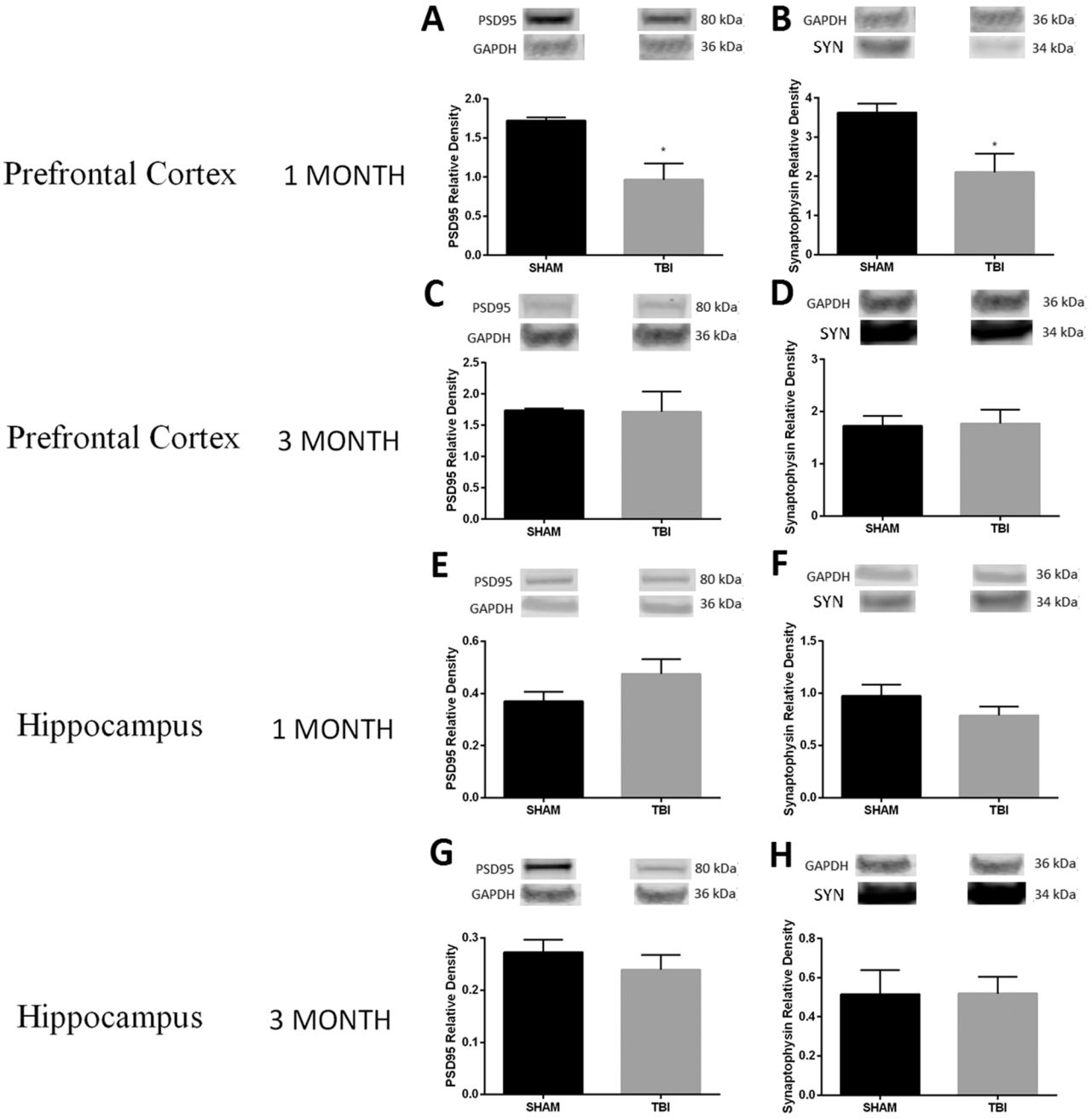

Fig. 6. Synaptic structural damage was assessed by post-synaptic density 95 (PSD-95) and synaptophysin markers. Western blot images of PSD-95 and synaptophysin markers as well as GAPDH (housekeeper protein) at the A–D) PFC and E–H) hippocampus for each of the time point. The graphs illustrate the relative density of A,C,E,G) PSD-95 and B,D,F,H) synaptophysin in TBI animals when compared to sham in the PFC at A–B) 1 month and C–D) 3 months post-injury, and in the hippocampus at E–F) 1 month and G–H) 3 months post-injury. Graph represent the mean ± SEM, (n = 5 per group; *p < 0.05 compared to shams).

task [33,34], similar to the deficit seen here, with post-TBI animals taking longer to locate the escape box when it was moved during the probe trial. These deficits were associated with decreased levels of PSD-95 and synaptophysin within the PFC, suggesting synaptic dysfunction. Few studies have examined the effect of TBI on synaptic morphology in the PFC region post-TBI, with Hoskinson et al finding alterations in dendritic spine density at 4 months following a parietal CCI injury [35] and Zhao et al finding a significant reduction of dendritic spine density in layer II/III pyramidal neurons of the medial PFC at two weeks post-FPI [36]. This supports the idea that TBI can cause significant disruption to the PFC region. Notably, although PSD-95 and synaptophysin had returned to sham levels by 3 months post-injury, functional deficits persisted, suggesting that there may have been persistent alterations in the circuitry (ex: serotonin circuitry) or changes in the functionality of the neurons (ex: receptor expression) of the PFC post-TBI. Interestingly a study by Park and Friston suggest that some functional outcome such as task-orientated cognition may be resulted from a divergence in structural and functional networks in the brain [37]. This implies that although structural networks may be recovered from injury (as seen in our study at 3 months), impairment in the functional networks (not investigated) may drive the persistent impairment seen. Further studies on the dynamics of these two networks in relation to cognition may provide better insight to post-TBI studies as well as neurodegeneration studies. Besides that, specific examination of synaptic morphology within different layers and specific regions of the PFC may provide further insight into circuitry alterations. It might also be beneficial to

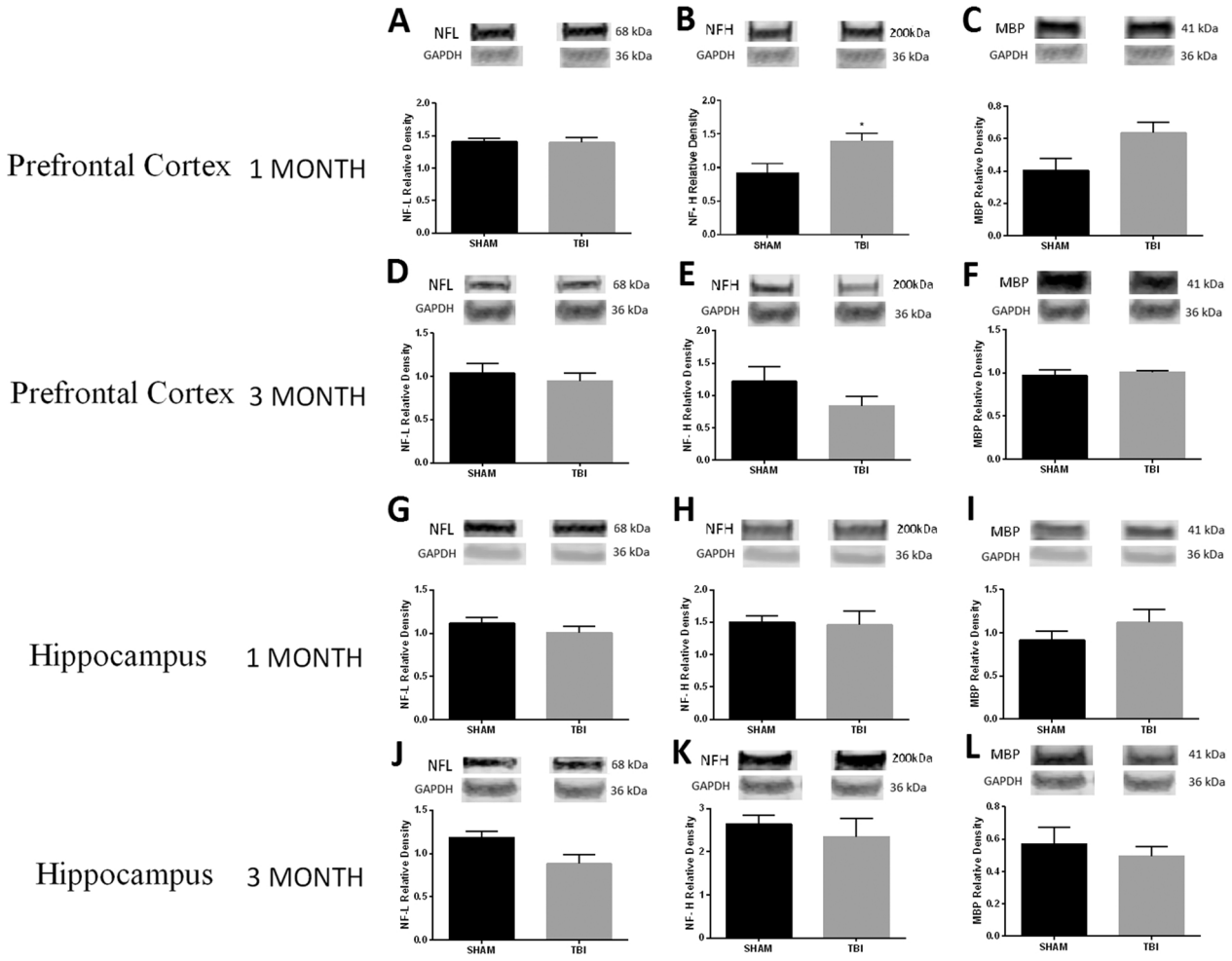

Fig. 7. Neuronal structural damage was assessed by neurofilament-light chain (NF-L), neurofilament-heavy chain (NF-H) and myelin basic protein (MBP) markers. Western blot images of NF-L, NF-H and MBP markers as well as GAPDH (housekeeper protein) in the A–F) PFC and G–L) hippocampus for each of the time point. The graphs illustrate the relative density of NF-L, NF-H and MBP in TBI animals when compared to sham in the PFC at A–C) 1 month and D–F) 3 months post-injury, and in the hippocampus at G–I) 1 month and J–L) 3 months post-injury. Graph represent the mean ± SEM, (n = 5; *p < 0.05 compared to shams).

investigate total neuron number in future studies, for a more precise measurement of synapse loss. Since persistent functional impairments were seen at 3 months, this could suggest there may be on-going neuronal network damage resulted from TBI (that may have branch from the structural damage seen at 1 month) which may drive early dementia or neurodegeneration.

As well as evidence of synaptic disruption, levels of NF-H were also significantly increased at 1 month post-injury within the PFC, before returning to baseline at 3 months, although no changes were noted in levels of NF-L. Neurofilaments are the dominant intermediate filament of axons [38,39] and are thought to be a key contributor to axon strength and resilience to mechanical stretch [40]. Immediately following diffuse impact acceleration and fluid percussion injuries, neurofilament compaction due to side-arm phosphorylation or proteolysis is known to be a key indicator of axonal integrity [41,42]. Activation of neuronal proteases is also associated with an acute reduction in levels of neurofilament as measured via western blot encompassing the light, medium and heavy subtypes [43,44]. The increase in NF-H at one month post-injury may therefore reflect a rebound reparative response following this acute injury phase involving disruption and loss of these proteins. Another potential explanation for the increase seen in NF-H in the current study is as a protective mechanism against toxic oxygen radical species. Wataya et al found that NF-H may act to sequester toxic lipid peroxidation byproducts in aldehydes, in order to protect critical active sites on proteins from oxidative attack [45]. NF-H is thought to preferentially perform this task as it is a lysine-rich protein, the component providing the buffering mechanism [46]. Unfortunately, within our study, we did not investigate oxidative stress markers directly, but instead used a measurement of superoxide dismutase 1 (SOD1), an antioxidant enzyme against superoxide radicals [47]. Although it can be argued that changes in SOD1 levels might be affected by the hypoxia insult during TBI induction, which was showed to be true in the hippocampus by Ramanathan et al., the study also showed that certain areas of the brain such as the cortex are resistant to hypoxia and therefore unaffecting the SOD1 levels [48]. Moreover, a recent study by Coimbra-Costa, showed that reoxygenation after acute hypoxia, as in our study, returned oxidative stress parameters and antioxidant enzymes to control or sham values suggesting the hypoxia treatment post-TBI may not affect the SOD1 levels in the brain [49]. Interestingly, our study found the levels of SOD1 were elevated, like those of NF-H, at 1 month post-injury within the PFC only, suggesting that this could be a similar protective mechanism against elevated levels of reactive oxygen species (ROS) resulted from the TBI. Indeed, overexpression of SOD1 is known to be neuroprotective in a number of models of brain injury [50,51]. Previous studies have shown ongoing oxidative stress within the injured parietal cortex at 1 month following FPI injury, as indicated by an increase in levels of oxidative damaged lipids and proteins [52,53]. Future studies should confirm whether there is evidence of ongoing oxidative stress within the PFC following a purely diffuse weight drop injury.

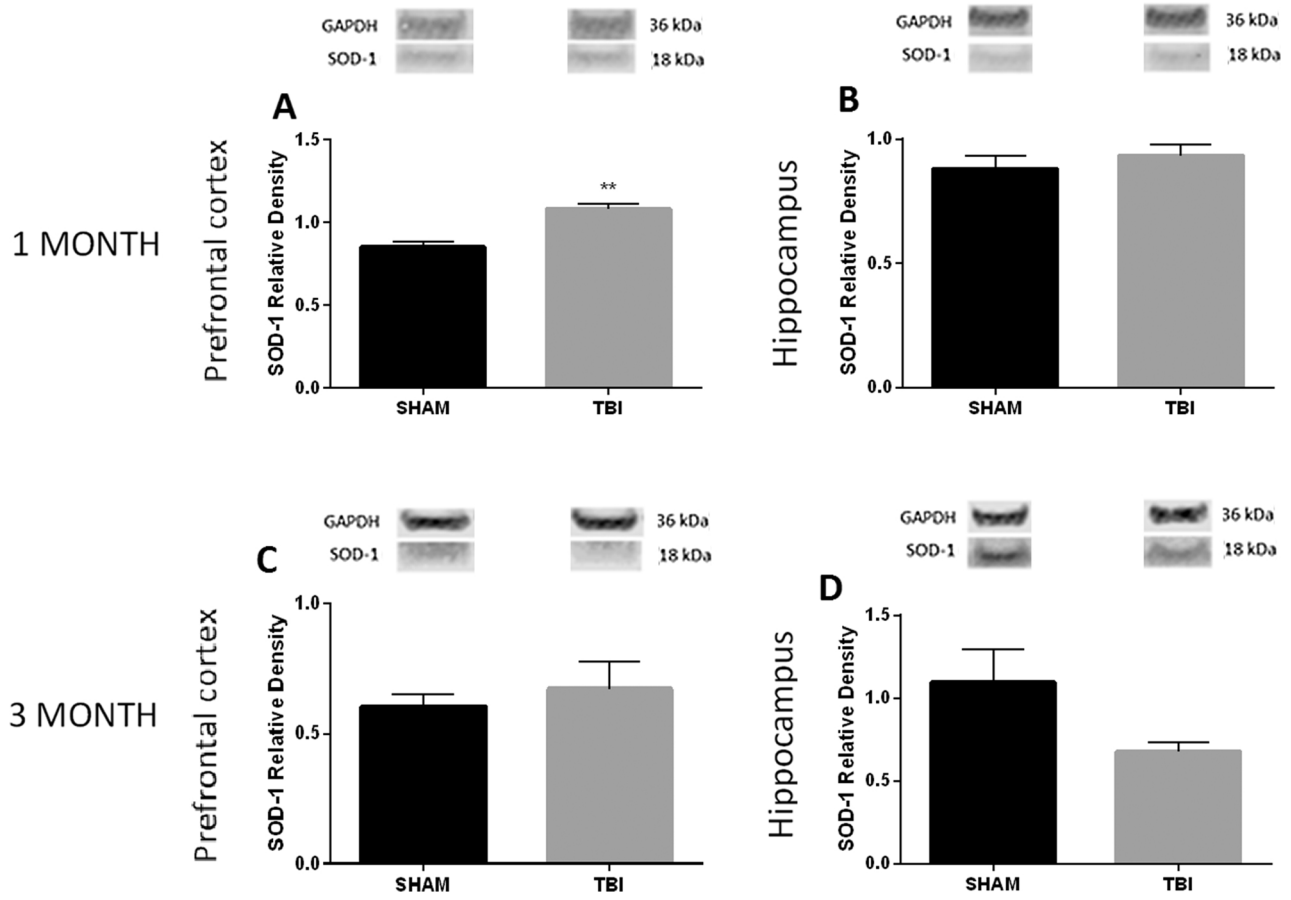

Fig. 8. Oxidative stress was assessed by superoxide-dismutase 1 (SOD-1) marker. Western blot images of SOD-1 and GAPDH (housekeeper protein) in the A,C) PFC and B,D) hippocampus for each of the time points. The graphs illustrate the relative density of SOD-1 in TBI animals when compared to sham in the PFC at A) 1 month and C) 3 months post-injury, and in the hippocampus at B) 1 month and D) 3 months post-injury. Graphs represent the mean ± SEM, (n = 5 per group; **p < 0.01 compared to shams).

Surprisingly, despite the pattern of behavioural deficits seen here and the evidence of synaptic dysfunction, increased neuroinflammation was not seen in the PFC at either 1 or 3 months post-injury. In fact, a reduction in the number of microglia and astrocytes was noted in this region at 1 month post-injury. Given that these cells have a number of beneficial functions, including release of neurotrophic factors, such as BDNF [54,55], modulation of neurotransmitter levels within the synapse [56] and supply of energy to neurons [57], this decrease may not be beneficial. Indeed, previous reports have found a decrease in levels of GFAP, a cytoskeletal protein expressed by many astrocytes, in the PFC of depressed patients [58–60]. It has been proposed that this alteration in astrocytes may influence glutamatergic signalling, thereby contributing to pathology [61,62]. The mechanism driving this decrease in resident immune cell numbers within the PFC at 1 month post-injury is not known, but it is possible that these cells may have migrated to other sites, such as the corpus callosum [63], with restoration of numbers by 3 months post-injury. Further studies are needed to confirm this result, as well as to assess if other anatomical regions are affected at these time points and later time points. Furthermore, as only total number of microglia were assessed, it is important to also confirm whether they are resting or reactive to provide a clearer picture of the neuroinflammatory reaction after injury. Neuroinflammation is significantly more complex than microglia or astrocytes alone. While this was beyond the scope of the current study, it is also important to assess

Table 1
Summary of behavioural results; changes in TBI when compared to shams at 1 month and 3 months post injury.

Test Paradigm	Behaviour measurement	1 month	3 month
Open Field Test (OFT)	Distance Travelled (m)	(Δ = − 2.99)	↓ (Δ = − 11.83)* p = 0.021
	Time in Center (s)	(Δ = 1.97)	(Δ = − 1.64)
Elevated Plus Maze (EPM)	Time in open arms (s)	(Δ = 8.29)	(Δ = − 15.17)
Forced Swim Test (FST)	Time Immobile (s)	↑(Δ = 26.57)* p = 0.027	↑(Δ = 34.45)** p = 0.004
Y-Maze	Novel Preference	(Δ = − 0.045)	(Δ = − 0.018)
Barnes Maze	Escape Latency to box on Acquisition Training (s)	NA	Day 1: (Δ = − 0.523) Day 2: (Δ = − 15.80) Day 3: (Δ = 3.697)
	Escape Latency to Old box (s)	NA	(Δ = − 13.78)
	Escape Latency to New Box (s)	NA	Trial 1: (Δ = − 38.95)* p = 0.014 Trial 2: (Δ = − 8.781)

Note: *p < 0.05, **p < 0.01, ↓ = decrease in value when compared to shams, ↑ = increase in value when compared to shams, Δ = (mean of TBI – mean of sham).

Table 2
Summary of histopathological results; changes in TBI when compared to shams at 1 month and 3 months post injury, at the prefrontal cortex and hippocampus region.

Markers	Prefrontal Cortex		Hippocampus	
	1 month	3 month	1 month	3 month
GFAP (glial fibrillary acidic protein)	↓ (Δ = − 57.60)* p = 0.019	(Δ = 0.59)	(Δ = − 14.50)	(Δ = − 8.17)
IBA1 (ionized calcium binding adaptor molecule 1)	↓ (Δ = − 20.70)* p = 0.049	(Δ = 6.15)	(Δ = − 20.20)	(Δ = 19.06)
PSD-95 (postsynaptic density protein 95)	↓ (Δ = − 0.76)* p = 0.039	(Δ = − 0.02)	(Δ = 0.11)	(Δ = − 0.03)
Synaptophysin	↓ (Δ = − 1.52)* p = 0.049	(Δ = 0.05)	(Δ = − 0.19)	(Δ = 0.003)
NF-L (neurofilament light chain)	(Δ = − 0.01)	(Δ = − 0.09)	(Δ = − 0.10)	↓ (Δ = − 0.30) p = 0.06
NF-H (neurofilament heavy chain)	↑ (Δ = 0.48)* p = 0.027)	(Δ = − 0.38)	(Δ = − 0.04)	(Δ = − 0.29)
MBP (myelin basic protein)	↑ (Δ = 0.23) p = 0.055	(Δ = 0.04)	(Δ = 0.21)	(Δ = − 0.08)
SOD-1 (superoxide dismutase 1)	↑ (Δ = 0.23)** p = 0.002	(Δ = 0.07)	(Δ = 0.05)	(Δ = − 0.42)

Note: *p < 0.05, **p < 0.01, ↓ = decrease in value when compared to shams, ↑ = increase in value when compared to shams, Δ = (mean of TBI – mean of sham).

levels of markers such as chemokines and cytokines in order to fully evaluate the effect of TBI on neuroinflammation [64]. Additionally, TBI has the capacity to lead to long-term alterations in neurochemical signalling, which may be significant contributors to persistent behavioural deficits following injury, so future studies should also investigate levels of key neurotransmitters, including dopamine and serotonin.

In contrast to the evidence of structural changes within the PFC at subacute time points post-injury, this study found minimal pathology within the hippocampus. This lack of hippocampal pathology is supported by the lack of deficits in the learning phase of the Barnes Maze or in recognition memory as assessed by the Y-Maze. These tasks preferentially assess hippocampal dependent learning with Conrad et al demonstrating that bilateral damage to the CA3, CA4 or dentate gyrus led to a decrease in spatial memory on the Y Maze [65]. In regards to the lack of hippocampal mediated cognitive impairment seen in this study, previous studies, contrastingly, have shown persistent cognitive deficits post-TBI, with, for example, Pearce et al observing significant deficits in spatial learning ability in the MWM beginning at two months and lasting up to one year following lateral FP brain injury [66], with similar reports of cognitive deficits from one month to one year following CCI injury [67]. This most likely relates to the more significant hippocampal damage associated with these injury models, with CCI associated with a 60% loss of hippocampal synapses acutely, that had still not recovered to pre-injury levels by day 60 [68]. Similar levels of significant hippocampal cell death have been reported following FPI [69], unlike the lack of synaptic damage seen here at either one or three months post-injury. Previous studies utilising the diffuse impact-acceleration model have similarly reported a lack of hippocampal dependent cognitive deficits on the MWM or radial arm maze [70,71], with a corresponding lack of neuronal loss within this area [70].

In conclusion, this study found that the PFC is significantly affected at one month following a diffuse TBI. There was evidence suggestive of both synaptic and axonal disruption that were associated with a decrease in the number of astrocytes and microglia. These alterations within the PFC also coincide with the impairments on the FST and decreased cognitive flexibility seen after injury. In contrast, the hippocampus was relatively spared at 1 and 3 months post-injury, with future studies needing to examine later time-points to determine if hippocampal damage emerges. Nevertheless, our study provides evidence of early structural changes in the prefrontal cortex after moderate-severe diffuse TBI. While these changes are resolved at 3 months post-injury, future studies should investigate whether they re-emerge or progress to other areas, such as the hippocampus, at later time points, which may contribute to long-term deficits or even predispose individuals to the development of dementia and other neurodegenerative conditions known to be linked to TBI.

4. Experimental procedure

4.1. Animals

Adult male Sprague-Dawley rats (10–12 weeks) (were used under approval of the University of Adelaide Animal Ethics Committee (M-2015-027). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery or moderate, diffuse TBI, with one subset subject to a functional assessment battery at 1 month post-injury (shams n = 14; TBI n = 19) and another at 3 months post-injury (shams n = 13, TBI n = 14). Following completion of functional assessment, animals were perfused and the brains collected for either histological or molecular analysis.

4.2. Injury model

The Marmarou impact-acceleration model [72] was utilized, as it has been extensively validated as a model of diffuse injury [19]. Animal weights ranged from 350 to 380 g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. They were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout. A midline incision was made to facilitate the placement of a metal disc centrally between lambda and bregma. Animals assigned to undergo TBI were then transiently taken off ventilation, strapped onto a foam, with injury induced by releasing a 450 g weight from a height of 2 m down a clear tube onto the centre of the metal helmet. Contact was observed to ensure single, direct impact without a rebound hit. Animals were then subject to hypoxic conditions (2 L/min nitrogen; 0.2 L/min oxygen) as previously described [63]. This is because this model of TBI leads to a period of apnea in unventilated animals which can lead to high mortality rates [73]. In order to regulate this animals are ventilated and then subjected to a hypoxic period to allow standardisation across the cohort, whilst still replicating the natural history of this injury type. Wound closure was performed with surgical staples. Successful induction of moderate to severe TBI was assessed 24 h later by rotarod scores of below 100, weight reduction of 5–10% and clinical signs (paresis and hunched posture). Any animal not falling within these parameters at 24 h post-injury was excluded from further behavioural and histopathological assessment. Based upon clinical record sheets, four animals were excluded from the study cohort after the TBI induction, from the moderate to severe TBI group as they did not meet the criteria of successful TBI induction. No additional distinctions were made between severity of injury. Shams assessed at the same timepoint (24 h) exhibited none of the clinical signs and had rotarod scores of more than 100. A previous study from our group has assessed motor performance for the first 7 days post-injury in this model [63]. In comparison to sham animals, TBI animals exhibited significant deficits in rotarod performance on days 1–3 post-injury, but this performance no longer

significantly differed from shams by 4 days post-injury.

4.3. Functional studies

Functional tests assessing cognition, anxiety, depression and motor function were performed at 1 month and 3 months post-injury. All functional data was recorded using the ANY-maze Video Tracking System version 4.99 m (Stoelting Co.). The functional tests were done in order from least to most aversive (stress inducing) except the rotarod test which was done at specific timepoints throughout the experiment regardless of other tests. All behavioural tests were conducted with the observer blinded to injury/sham status.

4.3.1. Rotarod

The rotarod is used as a standard motor coordination evaluation test for rodents [74]. Animals were placed on an elevated horizontal rod that rotates along the longitudinal axis. Animals were first habituated on the stationary rod for 10 ss. Then, for every 10 s thereafter, the rotation of the rod was accelerated at a constant rate of 3 rpm until the 100 s mark (maximum acceleration speed of 30 rpm). Animals were kept on the rotarod at the maximum speed for a further 20 s before decelerating the speed and removing the animal from the test. The rotarod score was measured by the latency of the animal to fall off the rod. Animals were trained for 3 consecutive days or until a score of 120 (baseline) was achieved. Following injury, animals were tested on the rotarod at 24 h then every 7 days following, i.e., day 8, day 15 and so on till the endpoint of the study.

4.3.2. Open field test

The open field test (OFT) is a common tests of locomotor activity [75]. Animals were placed in the centre of a large square box (95 cm × 95 cm) with walls at height 44.5 cm and the total distance travelled over a 5 min period was recorded.

4.3.3. Elevated plus maze

The elevated plus maze (EPM) is widely used in anxiety research [75]. Animals were placed in the centre of an elevated (50 cm in height) cross-shaped maze consisting of two open and two closed (walls of height 40 cm) maze arms (each of length 50 cm), facing the open arms, for 5 min. Time spent in the closed arms versus open arms was recorded, with increased time spent in the closed arms thought to represent anxiety-like behaviour.

4.3.4. Y-maze

The Y-Maze is used to test cognition in terms of spatial recognition memory [76]. In the Y-Maze, animals are placed in an equal angled Y-shaped arena, with each arm of the maze identical in size and shape but visually distinct (due to cues on the wall) from the others. The test involves two 3-minute trials separated by 1 h. In the first trial, one arm was closed off with a clear wall (novel arm) to enable the animal to visually recognise its presence; in the second trial, this novel arm became accessible (wall removed). In cases of reduced spatial reference memory, the animal spends less time within the novel arm.

4.3.5. Barnes maze

The Barnes maze evaluates spatial learning and memory in rats [77]. The maze is an elevated, open circular black platform with 18 holes evenly distributed along its edges. One of the holes is pre-determined as the escape hole with a black escape box placed below the hole. The Barnes maze test was preformed over the course of five days; three days of acquisition trials, a rest day (no interaction with the animals) and a probe day. During the acquisition days, animals were subject to two trials spaced 15 min apart. They were placed in the centre of the Barnes maze in a brightly lit room with the time taken for the animal to find and enter the escape box recorded. On day 5, the escape box was relocated to a new hole and two trials conducted 1 h apart. In trial 1, the time taken for the animal to reach the old position of the escape hole was recorded. In both trials, the time taken to locate and enter the newly relocated escape box was recorded as a measure of cognitive flexibility.

4.3.6. Forced swim test

The forced swim test (FST) is widely utilised to assess depressive-like behaviour [46]. The animal was placed within an inescapable glass cylinder filled halfway with 25 °C water, adjusted for the animal's length so that the hind legs does not touch the bottom of the cylinder, for 5 min. The time spent immobile was recorded as a measure of behavioural despair.

4.4. Tissue collection and processing

Animals were randomly assigned for further processing, either by molecular analysis or immunohistochemistry, during euthanasia. Animals that were to be used for molecular analysis were transcardially perfused with 0.9% saline and the brain dissected with the prefrontal cortex (PFC) and hippocampus taken (n = 5 per group). Samples were snap-frozen in liquid nitrogen before being stored at −80 °C. The samples were then homogenised via sonication in freshly prepared buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 140 mM 2-mercaptoethanol) with protease inhibitor cocktail (Sigma), 10 u L/mL aprotinin, leupeptin, pepstatin A and 10 mM PMSF. Each sample underwent 3 bursts of 10 s duration under a sonicator probe. Homogenised samples were centrifuged for 30 min at 14,000 rpm and 4 °C, before supernatant was collected. Protein concentration was estimated with Pierce BCA Protein Assay (ThermoScientific) at 750 nm absorbance.

Animals that were to be used for immunohistochemical analysis were transcardially perfused with 0.2 m L heparin + 10% formalin. Brains were removed and post-fixed in 10% formalin for 24 h, then blocked into 2 m m coronal sections and embedded in paraffin-wax. To examine the PFC, three consecutive 5 µm coronal slices were taken beginning at + 4.20 mm from Bregma for each animal. For hippocampal sections, three serial 5 µm coronal slices per animal were taken starting at -1.60 mm representing anterior hippocampus, −2.80 mm representing mid hippocampus and at −3.80 mm representing posterior hippocampus. Tissue mounted slides were allowed to dry at 37 °C overnight.

4.5. Western blot

Gel electrophoresis was performed using Bolt 4–12% Bis–Tris Plus gels (Life Technologies) with 50ug of protein loaded per well. Gels were run at 150 V for 30–45 min, depending on the molecular weight of the protein of interest, and transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes × 5 min), stained with Ponceau S red solution (Fluka Analytical) (5 min) for protein visualisation, and washed with distilled water until removal of Ponceau had been achieved.

Membranes were incubated for 2.5 h with primary and secondary antibodies in 1X iBind solution using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations: mouse anti-post-synaptic density protein 95 (PSD-95) (1:1000, ab2723 or ab18258, Abcam), rabbit anti-synaptophysin (1:1000, ab32127, Abcam), mouse anti-myelin basic protein (MBP) (1:250, ab62631, Abcam), mouse anti-neurofilament (1:300, ab24574, Abcam), rabbit anti-superoxide dismutase 1 (SOD1) (1:1000, ab13498, Abcam), and the primary housekeeping antibody chicken anti-GAPDH (1:4000, ab83956, Abcam). Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. Western blots were imaged using an Odyssey Infrared Imaging System

Table 3
Primary antibodies investigated using immunohistochemistry.

Primary Antibody	Analysis Target	Antigen Retrieval	Host animal and Dilution	Manufacturer
GFAP	Astrocyte reactivity	Citrate	Rabbit 1: 40,000	DAKO
Iba1	Microglial reactivity	Citrate	Rabbit 1: 20,000	Wako

[GFAP: Glial Fibrillary Acidic Protein, Iba1: Ionized calcium Binding Adaptor molecule 1].

(model 9120; software version 3.0.21) (LI-COR, Inc.) at a resolution of 169 µm. Semi-quantitative analysis of band signals was performed using ImageJ version 1.49 and Image Studio Lite version 5.2. Normalization of blot runs at 1 month and 3 month were performed using a single control sample of the respective time points. Thus, relative density of the samples were calculated based on the adjusted density for each blot, as below:

$$\text{Adjusted density} = \frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$$

$$\text{Relative density} = \frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$$

4.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed as per standard procedure. In brief, slides were oven-dried, de-waxed in xylene, rehydrated in ethanol and then placed into methanol with 0.5% hydrogen peroxide to block endogenous peroxidases. Then the slides were washed twice in phosphate buffered saline (PBS) and were blocked in normal horse serum (NHS) (1:30) for 30 min before incubation overnight with primary antibody (Table 3). The following day, slides were washed twice in PBS before application of secondary antibody (DAKO, 1:250, 30 min). Slides were once again washed twice with PBS, and then incubated with streptavidin peroxidase conjugate (SPC) (1:1000, 60 min). Slides were given a final wash in PBS, then incubated with 3,3′-Diaminobenzidinetetrahydrochloride (DAB) (1:50, 7 min) for antigen retrieval. Lastly, slides were counterstained with haematoxylin, placed in ethanol and subsequently in xylene, before mounting on cover slips.

Following staining, sections were scanned with Nanozoomer slide-scanner (Hamamatsu, Japan) and images viewed on NDPview (version 2). GFAP and Iba1 immunoreactivity was assessed quantitatively by counting the reactive and immunopositive cells per mm^2 within the hippocampus (CA1 + CA3 + DG region) and PFC (prelimbic region). The experimenter was blinded to the experimental group during cell counting and counts were performed twice Numbers obtained for each of the two counts were correlated to assess inter-attempt variability. This resulted in an r-value of 0.771.

4.7. Statistics

Except where outlined below, all data was analysed via two-tailed unpaired t-test using GraphPad Prism software. A repeated two-way analysis of variance was performed on the rotarod scores and on the acquisition days of the Barnes maze test. P values < 0.05 were considered statistically significant.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contribution statement

AA, JT and HC were involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

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Research report

Cognitive and neuropsychiatric impairments vary as a function of injury severity at 12 months post-experimental diffuse traumatic brain injury: Implications for dementia development

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ABSTRACT

Traumatic brain injury (TBI) is a common risk factor for later neurodegeneration, which can manifest as dementia. Despite this, little is known about the time-course of development of functional deficits, particularly cognitive and neuropsychiatric impairments, and whether these differ depending on the nature of the initiating insult. Therefore, this study investigated long term functional impairment at 12 months post-injury following diffuse TBI of different severities. Male Sprague-Dawley rats (420–480 g; 10–12 weeks) were either given a sham surgery (n = 14) or subjected to Marmarou's impact acceleration model of diffuse TBI for a single mild TBI (n = 12), repetitive mild TBI (3 mild diffuse injuries at 5 day intervals) (n = 14) or moderate to severe TBI (n = 14). At 12 months after injury, they were tested on a functional battery encompassing motor, neuropsychiatric (anxiety and depressive-like) and cognitive function. Our results showed that moderate to severe TBI animals exhibited significant impairments in cognitive flexibility (p = 0.009) on the Barnes maze when compared to age-matched sham animals. Neither repetitive mild TBI nor single mild TBI animals showed significant functional impairments when compared to shams. Thus, this study provides the first insight into chronic functional impairments associated with different severities of diffuse TBI, with moderate to severe TBI being a higher risk factor for impaired cognitive function at 12 months post-injury. Taken together, this may have implications for risk of dementia development following different severities of injury.

1. Introduction

Traumatic brain injury (TBI) covers a broad spectrum of disease, ranging from milder concussive insults to severe injuries. Since the first documentation of TBI leading to the development of parkinsonian-like symptoms in professional boxers [1], the research community has regarded TBI as not just a single insult, but as an injury that has ongoing functional consequences [2]. Repetitive mild (rmTBI) is linked with the later incidence of depression [3,4], anxiety [5] and impairments in learning and memory [6,7]. Studies on contact-sport athletes have associated a history of multiple concussions to a range of behavioural abnormalities, memory deficits and even parkinsonism [8] in later years. Conversely, following a single severe injury, cognitive impairments are most notable, with emergence of deficits in different cognitive domains over time, even in the subacute phase [9]. For example, serial neuropsychological testing over 5 years following injury found that 30% of patients who had experienced a moderate/severe TBI had clinically significant decline in two or more domains of cognitive functioning [9]. Higher rates of anxiety and depression are also reported chronically following a single moderate/severe injury, with reports of clinically significant depression in 46% of individuals at 10 years post-injury [10], compared to ~20% in the general population [11].

Experimental models of TBI also support the persistence of functional deficits following injury [12–14]. Animals subjected to a focal TBI induced by the controlled cortical impact model demonstrated persistent subtle cognitive deficits on the Morris Water Maze at 12 months post injury [15]. Similarly, animals injured via fluid percussion (FPI), which produces a mixed focal and diffuse injury, also had persistent cognitive deficits at 12 months post-injury [16,17]. Following purely diffuse axonal injury (DAI), cognitive deficits, evidenced by impaired spatial and recognition memory on the Barnes Maze, as well as increased anxiety, have been reported at 3 months post-rmTBI [13], while impaired spatial learning and cognitive flexibility and increased

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depressive-like behaviour were observed at 3 months following a single moderate/severe diffuse TBI [18]. To date, however, the behavioural effects of a purely diffuse injury have not been investigated pre-clinically beyond this 3 month time-point. This represents a significant gap in the existing literature, as "pure" forms of focal injury occur in only 28% of moderate-severe TBI cases, while diffuse axonal injury is seen in 72% of individuals, with "pure" diffuse axonal associated with significantly lower scores on the Glasgow Coma Scale [19].

These persistent functional impairments seen following injury may set the stage for later pathology, including a significantly increased risk for the development of neurodegenerative diseases, such as Alzheimer's (AD) [20–22], Parkinson's (PD) [23–25], chronic traumatic encephalopathy (CTE) [8,26,27], fronto-temporal dementia (FTD) [28,29] and motor neuron disease (MND) [30,31], as reviewed in [32–35]. Of these, the link between TBI and the later emergence of dementia has received the most attention to date. A dose-response relationship is thought to exist in terms of the risk of developing neurodegenerative disease [21], with more severe injury associated with greater risk, but even a single mild TBI may be linked to an increased risk of dementia [36]. A retrospective study utilizing health data from emergency department visits showed an increased risk of dementia with a minimum hazard ratio of 1.46 in moderate to severe TBI patients and a minimum hazard ratio of 1.1 in mild TBI patients, over a 5–7 year follow-up period [37]. A similar risk was reported in a Taiwan-based retrospective cohort study, in which individuals who had experienced a moderate to severe TBI showed a 1.68 fold higher risk of dementia than non-TBI patients [38].

Interestingly, the type of dementia that develops may differ depending on the nature of the initiating insult. For example, it is hypothesised based on case studies that a single moderate/severe TBI may be more strongly associated with accelerating age-related dementia, such as AD [20,39], while rm TBI may be more strongly linked to CTE [8]. However, the neuropathology following both single injury and rmTBI shares similarities, including the accumulation of hyperphosphorylated tau in the form of neurofibrillary tangles (NFTs) at the base of the sulci [40,41] and the development of a persistent inflammatory response post-injury [32,42,43]. Indeed, it has been suggested that TBI induced neurodegeneration may be its own unique entity, with further research needed into this question. Thus, this study aimed to document the range of chronic functional impairments, including in motor function, neuropsychiatric function and cognition, that may be associated with the different TBI severities (mild TBI, rmTBI TBI and moderate/severe TBI) at 12-months post injury in an experimental model of DAI. Furthermore, the study assessed whether functional changes at 12-months post-injury were associated with alterations in either neuronal number or integrity in the prefrontal cortex (PFC), a key region for cognitive function.

2. Results

2.1. Locomotion assessment

General locomotor activity was assessed as distance travelled (m) in the OFT. A one-way ANOVA showed a significant main group effect in the distance travelled in the OFT ($F_{3,50} = 3.234$, $p = 0.030$). However, post-hoc analysis showed no significant changes ($p > 0.05$) in distance travelled when the TBI groups were compared to shams at 12 months post-injury in the OFT. Nevertheless, the single mild TBI group did show significantly higher locomotor activity when compared to repetitive mild TBI animals (29.45 m ± 3.07 vs 18.68 m ± 2.09, $p = 0.021$) (Fig. 1A). This was confirmed by the generation of a heat-map showing activity within the OFT (Fig. 1B), which shows much greater coverage of the apparatus in the single mild TBI animals. A similar pattern in locomotor activity was seen in the distance travelled in the elevated plus maze, with a significant main group effect ($F_{3,50} = 4.963$, $p = 0.004$) driven by the single mild TBI group having a significantly higher distance travelled when compared to both the repetitive mild TBI group (6.14 m ± 0.60 vs 3.69 m ± 0.32, $p = 0.006$) and the moderate/severe TBI animals (6.14 m ± 0.60 vs 3.82 m ± 0.62, $p = 0.01$) after post-hoc analysis (Fig. 1C). Locomotor assessment in the Y-maze showed a non-significant main group effect ($F_{3,50} = 2.114$, $p = 0.110$) (Fig. 1D).

2.2. Anxiety-like behaviour

Anxiety-like behaviour was assessed through various parameters in the open field and elevated plus mazes. Both assess different anxiety stimuli in the animal, with the open field assessing anxiety over open spaces and the elevated plus maze assessing anxiety over open spaces and height [44]. In the open field, animals showed no significant main group effect either in time spent rearing ($F_{3,50} = 2.17$, $p = 0.103$) or time spent in the centre of the field ($F_{3,50} = 0.716$, $p = 0.547$) (Fig. 2A and B).

However, in the elevated plus maze, there was a significant main group effect in the time spent in the open arms ($F_{3,48} = 3.984$, $p = 0.013$), as well as the number of open arm entries ($H = 14.48$, $p = 0.002$) and the number of crossings ($H = 12.14$, $p = 0.007$). The mild TBI group showed the least anxiety-like behaviour, with the most time spent in the open arms (95.3 s ± 17.44) and the highest number of both open arm entries 7.5 (5–14) and crossings 14 (9–28), when compared to other groups. These were not significant when compared to shams; time in open arm (95.3 s ± 17.44 vs 61.24 ± 10.66 in shams, $p = 0.248$), number of open arm entries (7.5 (5–14) vs 6 (1–17) in shams, $p = 0.944$) and number of crossings (14 (9–28) vs 12 (2–31) in shams, $p = 0.786$), but was significant only when compared to moderate-severe TBI; time in open arms (95.3 s ± 17.44 vs 31.01 s ± 7.6, $p = 0.007$) (Fig. 2C), number of open arm entries (7.5 (5–14) vs 3 (0–10), $p = 0.001$) (Fig. 2D) and number of crossings (14 (9–28) vs 6.5 (0–20), $p = 0.006$) (Fig. 2E). Indeed this pattern can be seen in the heat-map, which shows the average amount of time spent in each part of the elevated plus maze across the injury groups, with the single mild TBI animals showing the highest amount of time in the open arms (Fig. 2F).

2.3. Depressive-like behaviour

Depressive-like behaviour was assessed through the forced swim test. Animals showed no significant main group effect in immobility time ($F_{3,50} = 1.434$, $p = 0.244$) (Fig. 3A), latency to first immobility ($F_{3,50} = 0.443$, $p = 0.723$) (Fig. 3B) or number of immobility episodes ($H = 2.104$, $p = 0.551$) (Fig. 3C) at 12 months post injury.

2.4. Cognition

Cognitive outcome was assessed using the Y-Maze for spatial memory and Barnes maze for learning, memory (reference and working memory) and cognitive flexibility (ability to reprogram previously learned task) [45]. Our results showed no significant main effect in any of the Y-maze parameters between the TBI groups and shams at 12 months post injury; novel preference ($F_{3,50} = 1.234$, $p = 0.307$) (Fig. 4A), number of novel arm entries ($H = 4.848$, $p = 0.183$) (Fig. 4B) and latency to 1st novel arm entry ($F_{3,48} = 0.0703$, $p = 0.976$) (Fig. 4C), as confirmed via heat-map analysis, with all animals showing greater intensity of staining in the novel arm, indicating higher occupancy of this arm (Fig. 4D).

As for the Barnes maze, learning acquisition showed neither a significant group effect within the three days of trial acquisition ($F_{3,50} = 2.208$, $p = 0.099$) nor a significant interaction effect ($F_{6,100} = 1.036$, $p = 0.407$), but, as would be expected, showed a significant main effect of time (trial days) ($F_{2,100} = 64.57$, $p < 0.0001$) (Fig. 5A). Indeed, all groups showed a significant improvement in escape latency from day 1 to day 2 ($p < 0.05$), with no significant

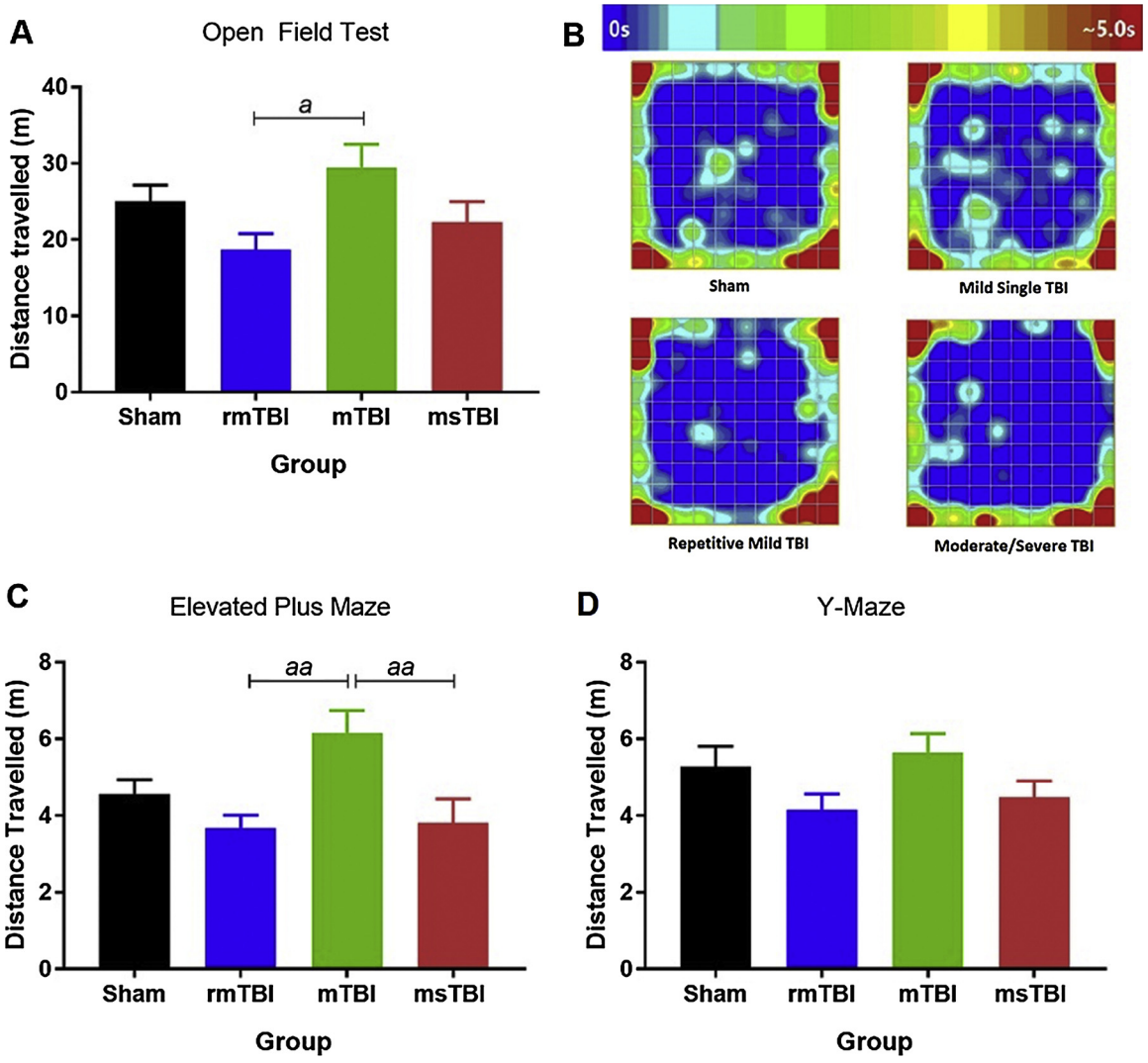

Fig. 1. Distance travelled (m) as a measure of locomotion on A) open field maze, C) elevated plus maze and D) Y-maze post injury. B) Heat map analysis on open field maze indicating location and exploratory time within the open field post injury. Graphs represent the mean ± SEM, (n = 12–14 per group; ^{aa} $p < 0.01$, ^{a} $p < 0.05$ compared between injury groups). Heat maps are from group composites.

differences noted between day 2 to 3 (Fig. 5A). On the heat-map, this can be seen as the more targeted time spent near the escape box on Days 2 and 3, compared to the more exploratory pattern on Day 1 (Fig. 6). There was also no significant main effect of group in latency to the old escape box location in trial 1 on probe day ($F_{3,38} = 0.346$, $p = 0.792$) (Fig. 5B). However, there was a significant group effect on cognitive flexibility, as indicated by time to find the new escape box, on probe day ($F_{3,38} = 4.343$, $p = 0.01$). In trial 1 on probe day, the moderate/severe TBI group had a significantly longer latency to reach the new escape box location when compared to shams (90.2 ± 13.44 s vs 48.08 ± 6.96 s, $p = 0.009$) (Fig. 5C), which was also seen in trial 2 on probe day, but which did not reach statistical significance (57.5 ± 12.25 s vs 28.5 ± 5.07 s, $p = 0.121$). This is illustrated by the construction of a heat-map showing the average time spent in each part of the Barnes Maze across the two trials, with the moderate/severe TBI animals spending more time in the old escape box location (Fig. 7). Similar to the acquisition trial, there was no significant interaction effect on the probe day ($F_{3,38} = 0.724$, $p = 0.544$) but there was a significant main effect on time (difference between trials) ($F_{1,38} = 27.93$, $p < 0.0001$), with all animals improving their escape latency from trial 1 to trial 2 ($p < 0.05$). Revisits to the old escape box location in trial 2 on probe day only showed a trend towards significance in effect between groups ($H = 7.403$, $p = 0.06$) (Fig. 5D). There was also no significant main effect seen between the groups in terms of reference memory error ($F_{3,38} = 0.932$, $p = 0.435$) (Fig. 5E) or working memory error ($F_{3,35} = 0.492$, $p = 0.69$) (Fig. 5F) on the probe day. Repetitive mild TBI and mild TBI groups showed no significant cognitive impairment when compared to shams on any of the cognition parameters ($p > 0.05$).

2.5. Molecular analysis

NeuN was used to assess whether TBI led to loss of neurons at 12-months post-injury in the PFC. There were no significant changes in the total number of neurons observed in the PFC ($F_{3,21} = 2.329$, $p = 0.104$) (Fig. 8A). This was further probed using several markers, including synaptophysin for assessing synaptic integrity, neurofilament light chain (NF-L) for assessing neurofilament structure and axonal stability and myelin basic protein (MBP) for assessing neuronal myelination stability. There were no alterations in synaptophysin levels in the PFC ($F_{3,21} = 0.244$, $p = 0.865$) (Fig. 8B). Similarly, neither levels of NF-L ($F_{3,21} = 0.762$, $p = 0.528$) (Fig. 8C) nor MBP ($F_{3,21} = 0.473$, $p = 0.705$) (Fig. 8D) differed as a function of injury at 12 months post injury.

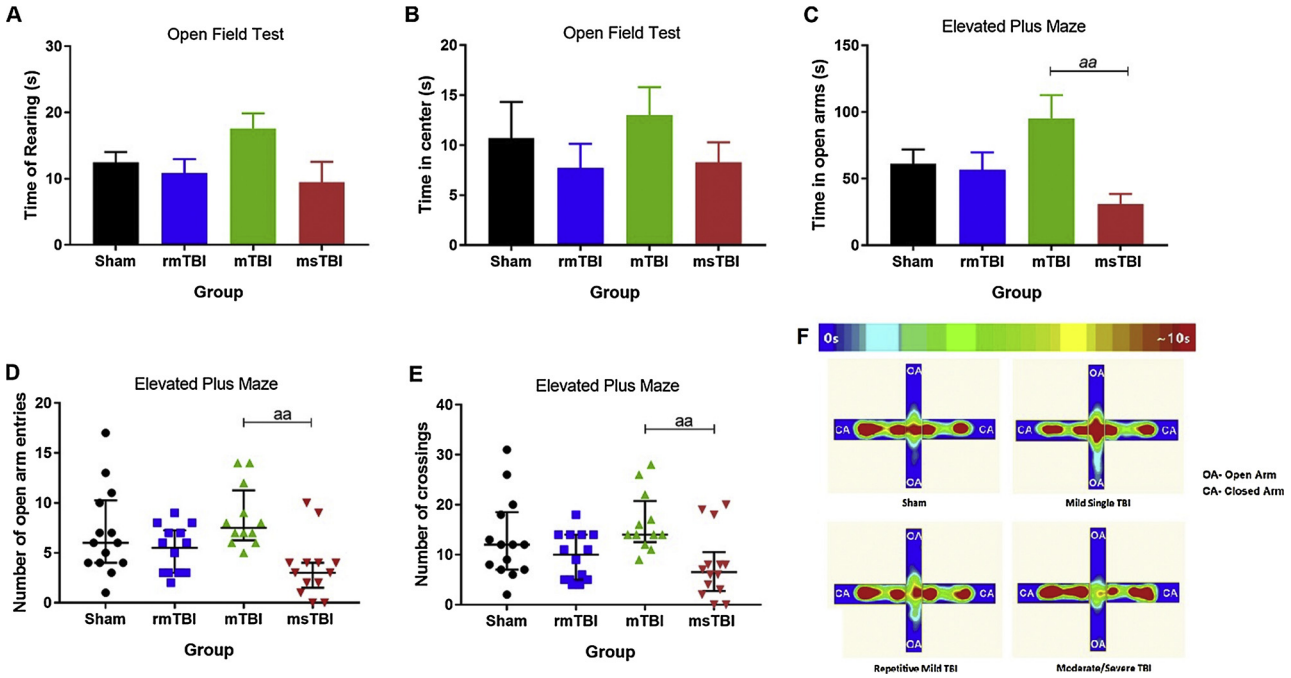

Fig. 2. Anxiety-like phenotype as measured by A) time spent rearing (s) and B) time spent in the centre (s) of the open field as well as measured by C) time in the open arms (s), D) number of entries into the open arms and E) number of crossings in the elevated plus maze post injury. F) Heat map analysis on elevated plus maze indicating location and exploratory time within the arms of the elevated plus maze post injury. Graphs represent the A-C) mean ± SEM and D-E) median with interquartile range, (n = 12–14 per group; ^{aa} $p < 0.01$ compared between injury groups). Heat maps are from group composites.

3. Discussion

The current study investigated the presence of functional impairments at 12 months post- TBI of different severities; mild TBI, mild repetitive TBI and moderate/severe TBI. At 12 months post-DAI, when compared to age-matched sham animals, neither impairments in general locomotor activity, the expression of depressive-like behavior nor impairments in cognition in terms of spatial learning, working memory or recognition memory were evident, regardless of TBI severity. However, the moderate/ severe TBI animals exhibited significant subtle impairments in cognitive flexibility when compared to shams. There was also a trend towards reduced anxiety, as evidenced by more time spent in the open arm of the EPM, in the single mild TBI group, with significant differences between this group and both the repeated mild and single moderate-severe TBI animals, although no differences were seen in comparison to sham-injured animals. This was further reflected in locomotor activity, with mild TBI animals having higher levels of activity compared to both repetitive mild TBI and moderate/severe TBI animals. Given the subtle alterations in cognitive flexibility at 12-months post-moderate/severe TBI, we used Western blot to analyse whether these were associated with changes in neuronal number or morphology in the PFC. Interestingly, no changes in either neuronal number or neuronal/synaptic integrity were found in the PFC at 12-months post-injury in any of the experimental groups. Taken together, the results of the current study seem to suggest that brain injury during early life has minimal effect on mid-life motor, cognitive or neuropsychiatric function, although subtle impairments in cognitive flexibility may still set the stage for the later emergence of more significant behavioural impairment.

The most notable finding in this study was that moderate-severe TBI led to a subtle impairment in cognitive flexibility at 12 months post-injury, with no effect seen on either spatial or recognition memory. This may indicate preferential disruption of prefrontal cortex function, as this region is critical for executive function, which governs cognitive flexibility [46,47]. Indeed, TBI has been consistently identified as a risk factor for higher-order cognitive deficits involving the frontal and prefrontal cortices [48]. In healthy adults, tasks like the Trail Making Test-B, which require cognitive flexibility and switching attention, lead to activation of the dorsolateral prefrontal (DLPFC) and medial prefrontal regions of the brain [49]. Following TBI, performance on Trail Making Test-B, as well as other measures of cognitive flexibility, attention and working memory, such as the Hayling, Selective Attention

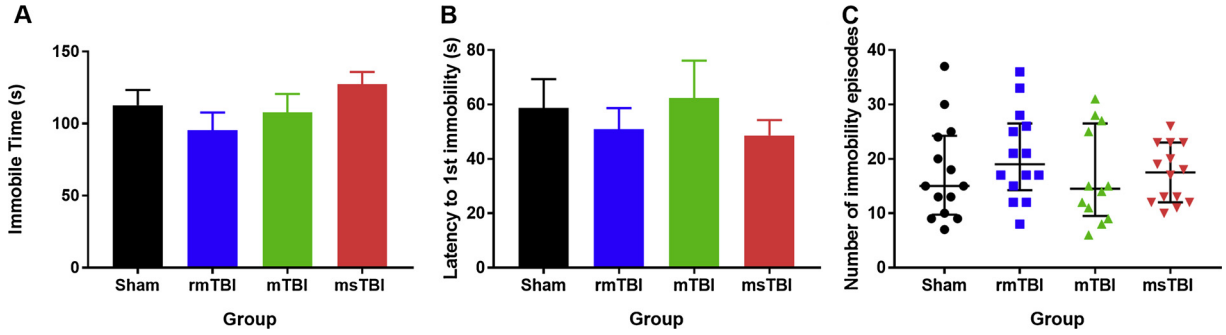

Fig. 3. Depressive-like behaviour as measured in forced swim test by A) time spent immobile (s), B) latency to first immobility (s) and C) number of immobility episodes post injury. Graphs represent the A-B) mean ± SEM and C) median with interquartile range, (n = 12–14 per group).

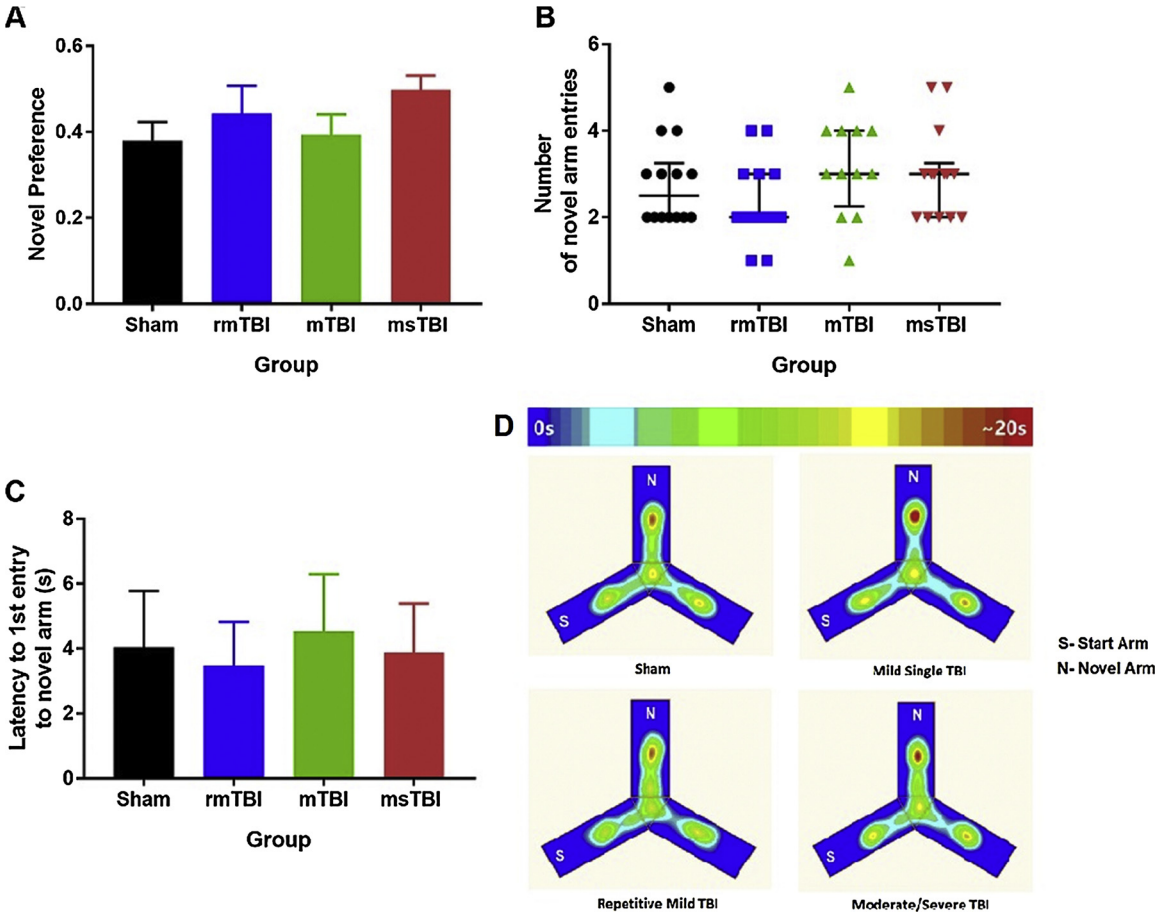

Fig. 4. Cognition assessed through Y-maze for spatial working memory measured by A) novel preference, B) number of entries into the novel arm and C) latency to first novel arm entry post-injury. D) Heat map analysis on Y-maze indicating location and exploratory time within the arms of the Y-maze post injury. Graphs represent the A & C) mean ± SEM and B) median with interquartile range, (n= 12-14 per group). Heat maps are from group composites.

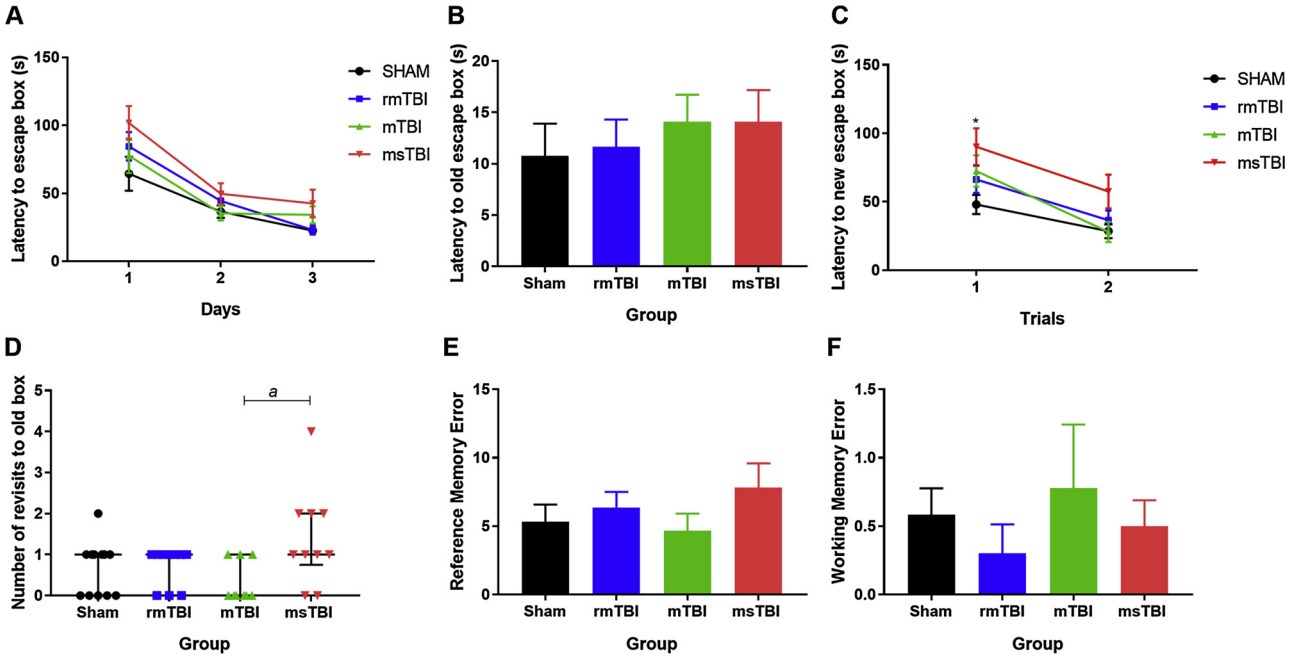

Fig. 5. Cognition post-injury assessed through Barnes Maze for learning measured by A) latency to escape (s) on acquisition day, for memory measured by B) latency to old box location (s) on trial 1 probe day, D) number of revisits, E) reference memory error and F) working memory error on trial 2 on probe day, as well as for cognitive flexibility measured by C) latency to escape to the new box (s) on probe day. Graphs represent the A-C, E-F) mean ± SEM and D) median with interquartile range, (n= 12–14 per group). [a] $p < 0.05$ compared between injury groups, [*]$p < 0.05$ compared to shams).

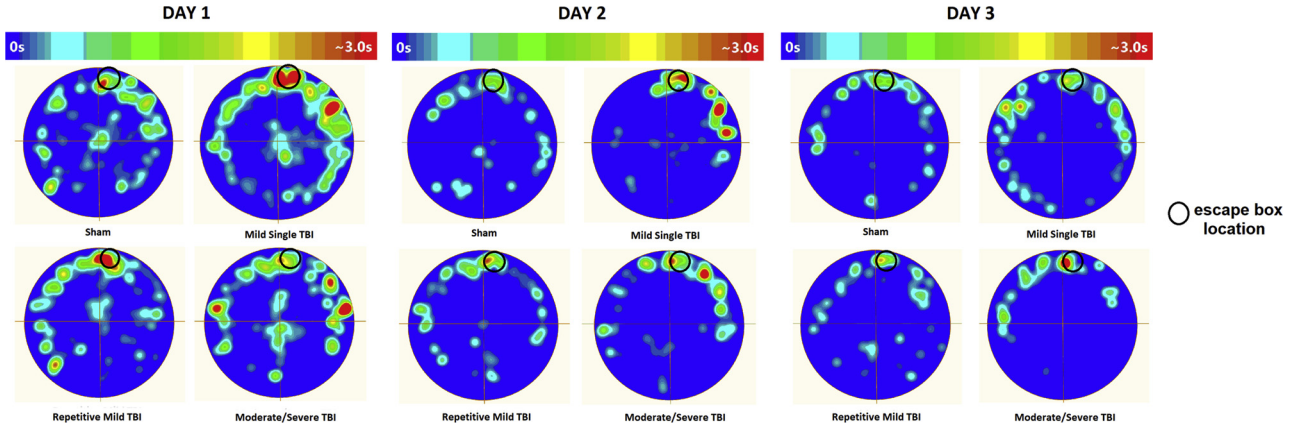

Fig. 6. Heat map analysis on Barnes maze indicating location and exploratory time within the Barnes maze on acquisition days post injury. Heat maps are from group composites.

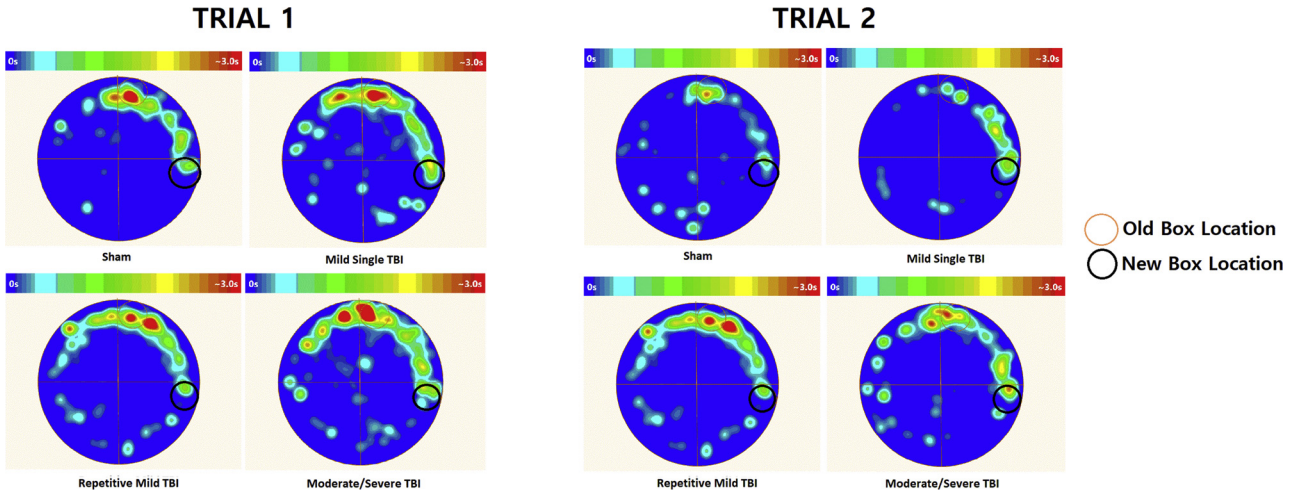

Fig. 7. Heat map analysis on Barnes maze indicating location and exploratory time within the Barnes maze on probe day post injury. Heat maps are from group composites.

Task, n-back and Symbol Digit Modalities Test, is slowed [50]. This slowed information processing speed post-TBI is associated with lower fractional anisotropy (FA) and higher mean diffusivity (MD) scores, indicating white matter abnormality, in the majority of tracts assessed [50]. Consistent with this, Ware and colleagues (2018) recently demonstrated that veterans who have suffered blast-induced TBI display elevated quantitative anisotropy (QA) and reduced right hemisphere volume in all subcortical-DLPFC tracts assessed, with decreased fibre count in the right-DLPFC-putamen tract and increased generalized FA in the right DLPFC-thalamus tract specifically [51].

Similar effects on cognitive flexibility and prefrontal cortex function following a single moderate/severe TBI have also been reported preclinically [52]. In a model of lateral fluid percussion, working memory, as assessed by a T-maze task, was significantly impaired up to one week post-TBI, an effect that was accompanied by alterations in prefrontal cortex function [53]. Similarly, previous work from our group has shown impairments in cognitive flexibility at 3 months following a DAI [18]. More chronically, in a CCI model that produced frontal contusions, impairments in reversal learning were observed at 12 months post-injury in a rule shift assay, a measure of cognitive flexibility [54]. It is not known, however, whether this deficit persisted from the time of injury or emerged at some later time-point prior to testing at 12 months, with further studies needed to incorporate a temporal time-course of behavioural changes required.

Despite the subtle alterations in cognitive flexibility observed in this study, there were no changes in the PFC in either total neuronal number, as measured by NeuN, or neuronal morphology, as measured by levels of synaptophysin, NF-L or MBP, at 12 months post-moderate/severe injury. This is consistent with earlier findings from our group, which showed no changes in neuronal morphology in the PFC at 3 months following moderate/severe TBI in the same experimental model of DAI [18]. However, it is important to note that we conducted only a gross characterisation of neuronal morphology changes using WB analysis of total level of protein for each marker of interest. It is possible that a more in-depth analysis using IHC or neuroimaging techniques would have detected subtle changes in neuronal morphology or circuit connectivity, which are more likely to be present than gross alterations. For example, previous work in the lateral cortical impact model has demonstrated working memory dysfunction without the presence of neuronal cell death in the prelimbic region of the medial PFC [55], suggesting that more subtle alterations may drive these changes in PFC-mediated cognition. In support of this, Hoskison et al found significant shortening of layer V/VI basal dendritic arbours and an increase in the density of both basal and apical dendritic spines in the prelimbic region of the medial PFC of rodents at 4 months following a lateral cortical impact injury [56]. These subtle dendritic changes were accompanied by persistent alterations in working memory function on both delayed match-to-place and delayed alternation t-maze tasks [56]. Thus, future studies should investigate subtle alterations in neuronal morphology within different cortical layers and specific subregions of the PFC, as well as the connectivity of the PFC with downstream structures.

Interestingly, in contrast to the findings of the current study,

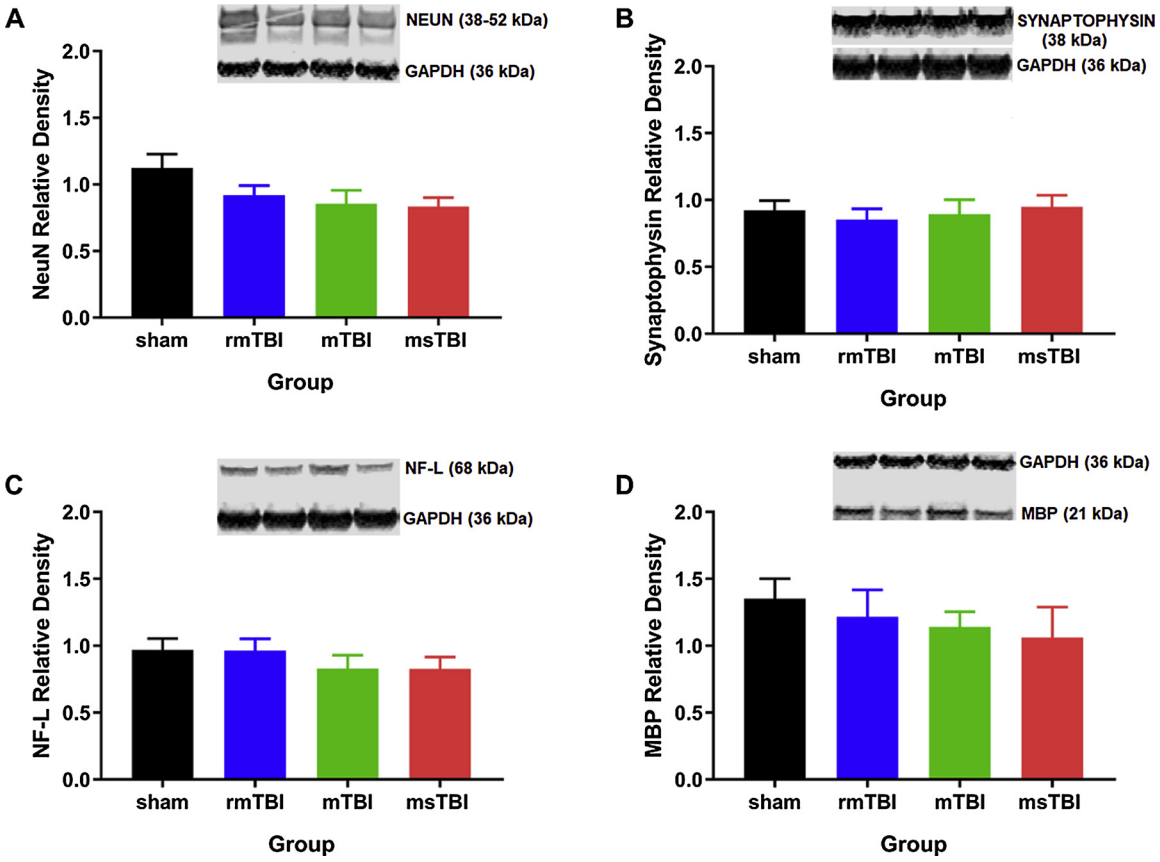

Fig. 8. Molecular analysis on the prefrontal cortex at 12 months was measured using semi-quantitative western blotting to analyse A) neuronal survival (total neurons, NeuN marker), B) integrity (synaptophysin marker) and C-D) structure damage (NF-L and MBP markers). GAPDH was used as a housekeeper protein for all analysis. Graphs represent the mean ± SEM. Representative images of the western blots were extracted from Image Studio Lite.

impairments in cognitive flexibility and alterations in PFC function have also previously been reported following rmTBI [48]. In the CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) model, a newly developed model of injury allowing precise control of injury direction and impact velocity, mice showed impaired in several PFC dependent functions, including social memory (27–28 days post-injury) and impaired spatial working memory (32–35 days post-injury) following five repeated mild hits [48]. These behavioural impairments were accompanied by a slight decrease in the adaptation rate of layer V pyramidal neurons in the mPFC [48]. Similarly, work from our own group has demonstrated mild cognitive impairments up to 3 months post-injury in a model of rmTBI [13]. However, it is important to note that neither of these studies investigated the long-term time point (i.e. 12 months) being investigated in this study. It is possible that impairments in executive function following rmTBI may steadily improve over time, normalizing by 12 months, and may worsen again later with ageing. Consistent with this theory, in individuals who have suffered a moderate/severe TBI, measures of executive control function improve over time in the first year post-injury, only to decline again from this point [57].

In contrast to the subtle alterations in cognitive flexibility demonstrated in the current study, other types of cognitive function, such as novel arm recognition in the Y-maze and learning the escape location in the Barnes Maze, known to be dependent on hippocampal functioning, were intact at 12 months post-injury in this model of DAI. Previous studies have shown that damage to the hippocampus through administration of kainic acid impairs performance on the Y Maze, with no preference seen for the novel arm [58], and that greater degrees of hippocampal loss following focal TBI are associated with worsening performance on the Barnes Maze [59]. Thus, the lack of impairment in these tasks seen in the current study would suggest an intact hippocampus. Indeed, previous studies of diffuse moderate-severe TBI have shown a lack of hippocampal cell loss [60] and preservation of hippocampal synaptic proteins [18], with a concomitant lack of hippocampal dependent cognitive deficits on either the MWM or radial arm maze following impact-acceleration TBI [60,61]. This is in contrast, however, to focal injury models, with cognitive impairment on the Morris Water Maze seen at 12 months post-CCI [15] and FPI [62], in line with the significant hippocampal damage induced following these injury types [58,63]. More recently, these hippocampal-dependent cognitive deficits have been shown to persist in mice up to 6.5 months post-injury following 3 impacts over 3 days in the CHIMERA model [64]. Given that TBI is associated with the later development of dementia, it may be that the 12 month time-point is insufficient to detect hippocampal deficits in a DAI model. Indeed, given that the animals are 14–15 months old at the conclusion of this study, this represents only later middle-age in humans, with perhaps more time needed to develop hippocampal pathology sufficient to lead to detectable cognitive deficits. Thus, future studies are needed to investigate whether subtle changes in neuronal morphology or connectivity may be present in the hippocampus at this chronic timepoint, even in the absence of overt behavioral change.

In the current study, no alterations in depressive-like behaviour were noted following any injury type, despite evidence to suggest that both rmTBI and moderate/severe TBI can increase the risk for depression [14,65]. Clinical studies show that there is a 40% prevalence of depression manifesting within a year post single moderate-severe TBI [4,66,67] and a three-fold increase risk of developing depression following repeated mild injuries [68]. Pre-clinical studies have also reported increased immobility time in the forced swim test up to 3 months following both a single moderate diffuse TBI [18,69] and rmTBI

(3 injuries in 10 days) [13,70], which suggests that this is a subacute deficit that has resolved by 12 months post-injury. Indeed, this is in line with other studies, with Jones et al (2008) reporting no behavioural despair (measured by FST) in their FPI animals at 6 months post injury [71]. Alternatively, the FST may not be an appropriate test to investigate depressive-like behavior at this time point, as the large size of the animals impedes swimming behaviour. Use of other measurements, such as the saccharin preference test or other test of depressive-like behaviours in rodents, may be needed to confirm the lack of depressive-like phenotype at chronic time-points.

Our study also demonstrated a lack of anxiety-like effect on both the OFT and EPM in the single moderate severe TBI animals and repeated mild TBI animals. Intriguingly, there was a trend towards decreased anxiety in the single mild TBI group in open arm time in the EPM, with significant differences between this group and both the repeated mild and single moderate-severe groups. This increased time in the EPM may be seen as decreased anxiety [44], or may reflect disinhibition or increased impulsivity [72]. Indeed, similar findings have previously been reported acutely following mTBI [48,73], suggesting that this may be a particular behavioural consequence of this type of insult that can persist to the chronic phase. The interpretation of these results as disinhibition or increased impulsivity may be supported by the increased locomotor activity noted in the single mild TBI animals, as they had a significantly greater distance travelled than the repetitive mild TBI animals in the OFT and had a significantly longer distance travelled than both of the other TBI groups in the EPM. This increased locomotor activity can be seen as hyperactivity [74–76], which is related to lesions in the cerebral cortex, mainly at the axis connecting the olfactory bulb and enthorhinal cortex within the pre-frontal cortex [77]. This hyperactive behaviour (increased distance travelled) on the open field has been reported previously after mild CCI in mice [75,76], supporting our findings. It is unclear why this phenotype was present in the single mild TBI and not the other injury groups, with the possibility that the difference could also reflect slight locomotor deficits in the repetitive mild TBI and moderate/severe TBI group. Nevertheless, since this behaviour was only present in the single mild TBI group and was not significantly different than shams, one must be careful to not over-interpret this result and future studies will be necessary to further investigate specific neuropsychiatric impairments at chronic timepoints post-TBI.

In conclusion, our study shows that a moderate/ severe diffuse TBI may lead to significant impairments in cognitive flexibility at 12-months post-injury, suggestive of potential subtle alterations in either the structure or connectivity of the PFC that must be confirmed with future studies. In contrast, hippocampal dependent tasks that rely on spatial recognition memory were unaffected in all injured animals, indicating potential preservation of this region at 12 months post-injury. Surprisingly, no long-term meaningful behavioural effects of either single or repetitive mild injuries were noted at 12 months post-injury. It is important to note, however, that the current study used all male rodents. Given the growing body of literature indicating differences in injury outcomes in males versus females, additional work is needed to determine whether the behavioural changes seen chronically following TBI present differently as a function of sex. Furthermore, future studies should investigate the extent to which the ageing process itself contributes to the emergence of cognitive change following TBI.

Taken together, the results of this study provide the first systematic comparison of the functional effects following different severities of diffuse TBI in a preclinical model of DAI at one year post injury. While behavioural effects were subtle at this timepoint, indicating that DAI in early life has minimal effect on mid-life function, regardless of initial injury severity, differences observed between injury severity groups may still provide meaningful information. The risk for impaired cognitive function may be greater following moderate/severe TBI than more mild forms of injury, which may have important implications for risk of dementia development following different severities of injury. This is particularly important given that it is still impossible to predict which individuals will go on to develop dementia following TBI. Thus, understanding the temporal profile of even subtle alterations in behaviour following different severities of TBI may have clinical utility in helping to determine risk profiles.

4. Materials and method

4.1. Animals

Male Sprague-Dawley rats (10–12 weeks) were used under the approval of the University of Adelaide Animal Ethics Committee (M-2015-243A) and (M-2015-187). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery (n = 7), repetitive sham surgery (3 incisions at 5 day intervals) (n = 7), a single mild diffuse TBI (n = 12), repetitive mild diffuse TBI (3 mild diffuse injuries at 5 day intervals) (n = 14), or moderate/severe diffuse TBI (n = 14). Animals underwent a comprehensive functional battery assessing motor, neuropsychiatric and cognitive function at 12 months post injury.

4.2. Injury model

The Marmarou impact-acceleration model [78] was utilized, as it has been extensively validated as a model of diffuse injury [79]. Animal weights ranged from 420 to 480 g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. Animals in the sham, repetitive sham, mild diffuse TBI and repetitive mild diffuse TBI groups were maintained on 2% isoflurane via nose cone throughout, while animals in the moderate/severe diffuse TBI group were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout [78,80]. A midline incision on the scalp was made to facilitate the placement of a metal disc centrally between lambda and bregma on the skull. Animals in the sham and repetitive sham groups received the incision only, with repetitive sham animals receiving the incision three times, with 5 day intervals between each incision.

Animals in the repetitive mild diffuse TBI and mild diffuse TBI group were removed from the nose cone and strapped onto a foam, with injury induced by releasing a 450 g weight from a height of 0.75 m down a clear tube onto the centre of the metal helmet; mild diffuse TBI animals receive this procedure only once, while repetitive mild diffuse TBI animals receive this injury three times, with 5 day intervals between each injury (Table 1). Conversely, animals in the moderate to severe diffuse TBI group were transiently taken off ventilation after incision, strapped onto a foam, with injury induced by releasing a 450 g weight from a height of 2 m (Table 1). Contact was observed to ensure a single, direct impact without a rebound hit in all animals. Only animals

Table 1
Injury model and specifications.

Injury Type	Weight of metal	Height of drop	Days of injury	Mechanically ventilated	Hypoxia Treatment	Saline Treatment
Repetitive Mild TBI	450 g	0.75 m	3 days (at 5 day intervals)	No	No	No
Mild TBI	450 g	0.75 m	1 day	No	No	No
Moderate to Severe TBI	450 g	2.00 m	1 day	Yes	Yes	Yes

in the moderate/severe diffuse TBI group were then subjected to hypoxic conditions (2 L/min nitrogen; 0.2 L/min oxygen) for 10 min, to replicate the clinical effects seen following this injury model without ventilation, as this hypoxic condition is known to exacerbate the severity of the injury [81,82]. Hypoxia alone had similar levels of cytoskeletal structure and neuroinflammation as shams under normoxic ventilation, as reported previously by Hellewell et al. [81]. Saline treatment (5 mL of 0.9% (w/v) saline solution) was administered subcutaneously to prevent dehydration [83] in the moderate/severe diffuse TBI group after wound closure, as well as if there was continuous weight loss post injury.

Wound closure was performed with surgical staples. Successful induction of moderate/severe TBI was assessed 24 h later by rotarod scores of below 100, weight reduction of 5–10% and clinical signs (paresis and hunched posture). Animals in the moderate/severe TBI group that did not meet the above criteria were excluded from the study. Moderate/severe TBI was associated with a 20% mortality rate due to brainstem haemorrhage, which is similar to other weight-drop model studies of moderate to severe TBI [84]. Shams, repetitive shams, mild diffuse TBI and repetitive mild diffuse TBI animals assessed at the same timepoint (24 h) exhibited none of the clinical signs outlined above and had rotarod scores of more than 100 s. Over the 12-month time period of the study, an additional 4 animals were lost due to age-related health complications.

4.3. Functional studies

Functional tests assessing cognition, anxiety, depression and motor function were performed at 12 months post-injury. All functional data was recorded using the ANY-maze Video Tracking System version 4.99 m (Stoelting Co.). The functional tests were done in order from least to most aversive (stress inducing) to the animals. The experimenter was blinded to the experimental groups of each animal throughout the duration of the study, with unblinding only occurring during analysis of the results.

4.3.1. Open field test

The open field test (OFT) is a common test of locomotor activity [85]. Animals were placed in the centre of a large square box (95 cm × 95 cm) with walls at height 44.5 cm and the total distance travelled over a 5 min period was recorded. Time in centre of the field and rearing time were also measured for anxiety-like behaviour.

4.3.2. Elevated plus maze

The elevated plus maze (EPM) is widely used in anxiety research [85]. Animals were placed in the centre of an elevated (50 cm in height) cross-shaped maze consisting of two open and two closed maze arms (walls of height 40 cm, each of length 50 cm), facing the open arms, for 5 min. Time spent in the closed arms versus open arms as measured by the centre point of the animal's body was recorded, with increased time spent in the closed arms thought to represent anxiety-like behaviour [85]. Other anxiety-like behaviour parameters measured in the EPM include number of centre crossings and number of open arm entries as measured by the centre point of the animal's body.

4.3.3. Y-Maze

The Y-Maze is used to test cognition in terms of spatial recognition memory [86]. In the Y-Maze, animals are placed in an equal angled Y-shaped arena, with each arm of the maze identical in size and shape, but visually distinct (due to cues on the wall), from the others. The test involves two 3-minute trials separated by 1 h. In the first trial, one arm was closed off with a clear wall (novel arm) to enable the animal to visually recognise its location; in the second trial, this novel arm became accessible (wall removed). In cases of reduced spatial reference memory, the animal spends less time within the novel arm [87].

4.3.4. Barnes maze

The Barnes maze evaluates spatial learning and memory in rats [88]. The maze is an elevated, open circular black platform of 1.2 m in diameter with 18 holes evenly distributed along its edges. One of the holes is pre-determined as the escape hole, with a black escape box placed below the hole. The Barnes maze test was preformed over the course of five days; three days of acquisition trials, a rest day (no interaction with the animals) and a probe day. During the acquisition days, animals were subject to two trials spaced 15 min. apart. They were placed in the centre of the Barnes maze in a brightly lit room, with the time taken for the animal to find and enter the escape box recorded. On day 5, the escape box was relocated to a new hole and two trials were conducted 1 h apart. In trial 1, the time taken for the animal to reach the old position of the escape hole was recorded. In both trials, the time taken to locate and enter the newly relocated escape box was recorded as a measure of cognitive flexibility. Number of revisits to the old box location on trial 2 of probe day, working memory error (measured as the number of revisits to the same hole after exploration of less than 3 different holes) and reference memory error (measured as the number of visits to any of the holes that was not the escape hole) were recorded as additional cognitive parameters.

4.3.5. Forced swim test

The forced swim test (FST) is widely utilised to assess depressive-like behaviour [89]. The animal was placed within an inescapable glass cylinder filled halfway with 25 °C water, adjusted for the animal's length so that the hind legs do not touch the bottom of the cylinder, for 5 min. The time spent immobile, number of immobile episodes and latency to first immobile episode were recorded as a measure of behavioural despair.

4.4. Tissue collection and processing

Animals were transcardially perfused with 0.9% saline and the brain was removed. The prefrontal cortex (n = 5–7 per group) was dissected and snap-frozen in liquid nitrogen before being stored at −80 °C.

The samples were taken out and homogenised in freshly prepared RIPA lysis buffer (150 mM sodium chloride, 50 mM Tris-hydrochloride acid of pH 7.5–8, 1% of NP-40 IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% of sodium dodecyl sulfate (SDS) and distilled water) with 1X cOmplete™ EDTA-free protease inhibitor cocktail (Sigma). After homogenisation, each sample underwent 3 bursts of 10 s duration under a sonicator probe with a cooling period between each burst. Then the samples were centrifuged for 30 min at 14,000 rpm and 4 °C, before the supernatant were collected. Protein concentration was estimated with Pierce BCA Protein Assay Kit (ThermoScientific) with the absorbance read at 540 nm. All supernatant were stored at −80 °C until further usage.

4.5. Western blot

Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus gels (Life Technologies) with 30 µg of protein loaded per well. Gels were run at 150 V for 1 h. After the run, blots were transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes × 5 min), stained with Ponceau S red solution (Fluka Analytical) (5 min) for protein visualisation, and washed with distilled water until sufficient removal of the Ponceau stain had been achieved.

Membranes were then incubated for 5 min with the 1X iBind solution before proceeding with the final step of simultaneous incubation with primary and secondary antibodies in 1X iBind solution for 2.5 h using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations; synaptophysin (1:4000, Abcam, ab32127), neurofilament light-chain (1:2000, Abcam,

ab72997), myelin basic protein (1:750, Abcam, ab62631) and NeuN (1:750, Abcam, ab177487) with housekeeper antibody GAPDH (1:1000, Abcam, ab83957 and 1:1000, Abcam, ab9485). Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. The blots were imaged using an Odyssey CLx Infrared Imaging System (model 9140) (LI-COR, Inc.) set at auto resolution for optimum visualisation. Semi-quantitative analysis of band signals were performed using Image Studio Lite version 5.2. Normalization of blot runs were performed using a single control sample (sham) across blots of the same protein of interest. Thus, relative density of the samples was calculated based on the adjusted density for each blot, as below:

$$\text{Adjusted density} = \frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$$

$$\text{Relative density} = \frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$$

4.6. Statistics

All data, with the exception of Barnes maze data, was analysed via one-way ANOVA (Analysis of Variance) with injury severity as the between subjects factor using IBM SPSS statistics 24 and GraphPad Prism software. A repeated two-way analysis of variance was performed on the acquisition days and latency to new escape box on probe day of the Barnes maze test, with trial as the within subjects factor and injury severity as the between subjects factor. Post hoc testing was conducted using Tukey's method. The Kruskal Wallis test was used for non-parametric measurements. For all tests, p values < 0.05 were considered statistically significant. Shams and repetitive shams were combined together as a single sham group, as there were no statistically significant differences in any parameters of behavioural and molecular data.

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Author contributions

AA was involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

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