Investigating the influence of risk factors on the cellular changes after mild traumatic brain injury

by

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Declaration of originality

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Statement of Ethical Conduct

"The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. Ethics Approval No A0015573 and A0016593"

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SUMMARY

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. TBI is most prevalent in people during their productive years and frequently results in prolonged or life-long motor and/or cognitive impairments, putting a significant economic burden on society. Mild TBI (mTBI), comprising over 80% of TBI cases, frequently results in acute neurosensory deficits and subacute ongoing neurological deficits with long-term implications, including the potential development of neurodegenerative diseases. Factors including the age at injury, sex, number of injuries and interval between injuries have the potential to affect pathological changes and the recovery process following mTBI, however, how these factors interact remains unclear. Therefore, the underlying cellular and subcellular responses evoked by mTBI need to be fully elucidated in order to understand how these factors affect outcomes following mTBI, and furthermore to develop effective therapeutic interventions. The current thesis is based on the hypothesis that specific cellular and subcellular responses (including neuronal and glial changes) differentially characterize pathological changes and recovery processes after mTBI.

This thesis investigated how the age at injury affects outcomes after mTBI. Studies focused on the cellular and subcellular reactions after injury. Studies found non-injured aged brains were characterized with cortical atrophy, higher astrocyte and microglial activation, as well as increased axonal degeneration compared to younger brains. Furthermore, using a mild lateral fluid percussion injury mouse model, results showed that although select cortical changes after injury were independent of the age at injury, astrocytic and microglial activation, as well as axonal degeneration after injury were age and post injury time interval dependent. This thesis suggests that the cellular and subcellular responses after mTBI are age dependent.

This thesis further investigated a potential drug intervention for mTBI. Neuronal cytoskeletal changes, including the morphological and functional changes of microtubules are recognized as a pathological feature of degeneration after TBI. Thus, I hypothesized that a microtubule stabilizing agent would have beneficial therapeutic potential following TBI. This study found that epothilone D was not toxic to cortical neurons and did not have any significant effects on the glial response in young and adult mice. With regards to axonal degeneration, in young mice that received a mTBI injury and subsequent EpoD treatment, measures of axon degeneration were also found to be similar compared to untreated animals. In older animals, however, the measures of axon degeneration were worse in injured animals after EpoD treatment compared to untreated injured animals. This thesis highlights the need for age dependent drug therapy for mTBI patients.

Finally, this thesis investigated how sex and an additional injury influence the outcome after mTBI. Conflicting literature exists regarding whether females have better outcomes than males in both experimental and clinical studies of TBI. Our results suggest that glial activation was distinctive between sexes, as well as different axonal degeneration between sexes with increased axonal degeneration after injury resolving earlier in females but exacerbated in male mice. Furthermore, our study also found a second injury, 48 hours following the first, significantly increased glial activation, as well as reduced the cortical thickness and exacerbated axonal degeneration after injury in male mice. This thesis suggested both sex and an additional injury differentially affected glial and axonal responses, which may further support the need for sex dependent and repetitive injury dependent clinical management of mTBI patients.

In summary, this thesis shows how risk factors including age at injury, sex and a second injury effected the cellular and subcellular reaction following mTBI in a mouse model. Furthermore, it suggests a potential need for age and sex dependent therapeutic interventions in mTBI patients.

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Table of Contents

ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANOVA	analysis of variance
APP	amyloid precursor protein
BBB	blood brain barrier
CBF	cerebral blood flow
CC	corpus callosum
CCI	controlled cortical injury
CCL2	C-C Motif Chemokine Ligand 2
CDC	Centers for Disease Control and Prevention
CNS	center nervous system
COX-2	cyclooxygenase 2
CRF	corticotropin-releasing factor
CT	computed tomography
CTE	chronic traumatic encephalopathy
DAI	diffuse axonal injury
DAPI	4', 6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EpoD	epothilone D
FJB	Fluoro-Jade B
GCS	Glasgow Coma Scale
GFAP	glial fibrillary acidic protein
HPA	hypothalamic-pituitary-adrenal
hr	hour
Iba1	Ionized calcium binding adaptor molecule 1

IC	internal capsule
IL	interleukin
iNOS	nitric oxide synthase
iPSC	induced Pluripotent Stem Cells
LFPI	lateral fluid percussion injury
LOC	loss of consciousness
min	minute
mTBI	mild traumatic brain injury
MRI	magnetic resonance imaging
NFs	neurofilaments
NR2B	N-methyl-d-aspartate receptor subtype 2B
PBS	phosphate-buffered saline
PD	Parkinson’s disease
PFA	paraformaldehyde
PPCS	persisting post-concussive symptoms
PSD-95	post synaptic density 95
PVN	paraventricular nucleus
rmTBI	repetitive mild traumatic brain injury
TAI	traumatic axonal injury
TBI	traumatic brain injury
TDP-43	TAR DNA-binding protein 43
TNF-α	Tumor Necrosis Factor α
YFP	yellow fluorescent protein

1. INTRODUCTION

Traumatic brain injury (TBI) is a non-congenital insult to the brain from an external mechanical force that can potentially lead to temporary or permanent impairment of cognitive, physical, and psychosocial functions (Syed et al., 2007). Most of the neurological impairments from TBI evolve over hours and days after the initial injury, which allows TBI to be divided into primary and secondary brain injury. Primary injury results from mechanical injury at the time of injury, and includes both focal and diffuse injuries. Secondary injury occurs hours or even days after the initial injury, and is caused by the physiologic responses to the initial impact (Al-Sarraj, 2016) (see Fig 1.1). TBI is a significant public health problem as it is a major cause of death and disability worldwide (Xu, 2017). It is reported that 50-60 million people worldwide (including at least 3.5 million in the US, and 2.5 million in Europe) are affected by TBI each year, the great majority of cases are mild TBI (Maas et al., 2017). In Australia, the overall incidence of TBI cases per 100,000 population is 99.1 in New South Wales (Pozzato et al., 2019), whilst in New Zealand, the total incidence of TBI per 100,000 person-years of is 749 cases for mTBI and 41 cases for moderate to severe (Feigin et al., 2013). TBI can occur at any age, with young children aged 0-4 years, older adolescents aged 15-19 years, and adults aged over 75 years having the highest incidence rates (Xu, 2017). TBI can be classified according to severity into mild, moderate or severe forms, with the vast majority of TBI cases being mild TBI (mTBI). The classification of mTBI has been a matter of controversy, which makes comparison of studies difficult (Lefevre-Dognin et al., 2020). In general patients, mTBI is diagnosed with a GCS score of 13-15. However, other clinical factors have been considered in the classification of mild TBI, such as loss of consciousness (Katz and Alexander, 1994), as well as the consideration of neuroimaging findings (Williams et al., 1990). The diagnosis of concussion resulting from a sporting injury is different, and depends on the severity of the neurologic dysfunction and the extent of the diagnostic testing (McCrory et al., 2017). Although the terms ‘mild TBI’ and ‘concussion’ are used interchangeably in trauma literature, it's still a debate as whether mTBI is synonymous with concussion or not, with ‘mTBI’ used in general medical contexts and ‘concussion’ more commonly used in the context of sports injuries (Greenwald et al., 2012). Clinically, mTBI is classified as an injury where post-traumatic amnesia or alteration of consciousness is less than or equal to 24hrs; the length of loss of consciousness is less than or equal to 30 min; and/or with Glasgow Coma Scale (GCS) 13-15 (Benzinger et al., 2009). mTBI is extremely common worldwide, with around 42 million people seeking medical attention for this condition each year (Gardner and Yaffe, 2015). Moreover, the financial burden of mTBI is extensive, with the cost of mTBI in the US military population alone rising extensively to around $250 million per year (McCrea, 2008). According to a population based study of TBI in New Zealand, the major cause of mTBI is falls (38%),

other causes include exposure to mechanical force (22%), transport accidents (19%), assault (17%) and other causes (4%) (Feigin et al., 2013). Populations involved in contact sports, military personnel and victims of domestic violence are at high risk for suffering mTBI (Gardner and Yaffe, 2015). Additionally, the estimates of the incidence of sport-related mTBI range from 1.6-3.8 million per year in the US (Langlois et al., 2006). Sex dependent incidence exists with regards to the susceptibility of TBI (Faul and Coronado, 2015), with women more likely to be injured during an assault or violence while men are most often injured due to falls and motor vehicle accidents (Iverson et al., 2011, Colantonio, 2016). Multiple injuries have been observed in individuals, particularly among war injured veterans and sport-injured athletes, which has resulted in repetitive mTBI (rmTBI) becoming an area of research focus.

Unlike moderate and severe TBI, individuals with mTBI often show cognitive impairments without identifiable tissue lesions or cavities in the cerebral cortex, even when using traditional neuroimaging techniques including magnetic resonance imaging (MRI) and computed tomography (CT) (Gao and Chen, 2011). Therefore, mTBI has been termed an invisible and silent epidemic. In the clinic, mTBI is typically characterized by a transient disturbance in brain function, with short-lasting neurological symptoms including headaches, dizziness and confusion (Carroll et al., 2004). Even though symptoms for the majority of patients subside within short-term after injury (Rivara and Graham, 2014), a proportion of patients may develop with persisting post-concussive symptoms (PPCS) (Vasterling et al., 2009, Anderson et al., 2017, Keenan et al., 2018), with long-lasting behavioural and cognitive deficits (Kraus et al., 2007). Additionally, mTBI may also be associated with an increased risk of developing neurodegenerative diseases later on in life, including Alzheimer's disease (AD) and Parkinson's disease (PD) (DeKosky et al., 2010). mTBI is an evolving and dynamic injury, in which injury forces induce physical stress in the brain that initiates a complex series of neurochemical and metabolic changes (Taber and Hurley, 2013). As it is shown in Fig 1.1, immediately after a brain insult, there are brain swelling; ischemia caused by decreased cerebral blood flow; excitotoxicity due to the release of excessive excitatory neurotransmitters; calcium-mediated brain damage, oedema and mitochondrial dysfunction that generates energy failure and excessive free-radical generation (Gupta and Przekwas, 2013). Moreover, injury also initiated necrotic and apoptotic neuronal cell death (Büki and Povlishock, 2006, Saatman et al., 2008). There are two main types of oedema, vasogenic and cytotoxic brain edema. Vasogenic edema relates to blood brain barrier (BBB) disruption, it occurs when the BBB is disrupted allowing an influx of plasma from the vasculature into the extracellular space (Peters and Vaughn, 1967). Cytotoxic edema is related with a failure in ATP-dependent multiple ion pumps (mainly Na+/K+-pumps) during energy shortage, it increases in water content within the intracellular compartment as a result of an osmotic gradient and cell swelling

(Kochanek et al., 2000). Although growing knowledge on the short and long-term behavioural and cognitive deficits after mTBI has been gained, the underlying mechanisms are still yet to be fully understood.

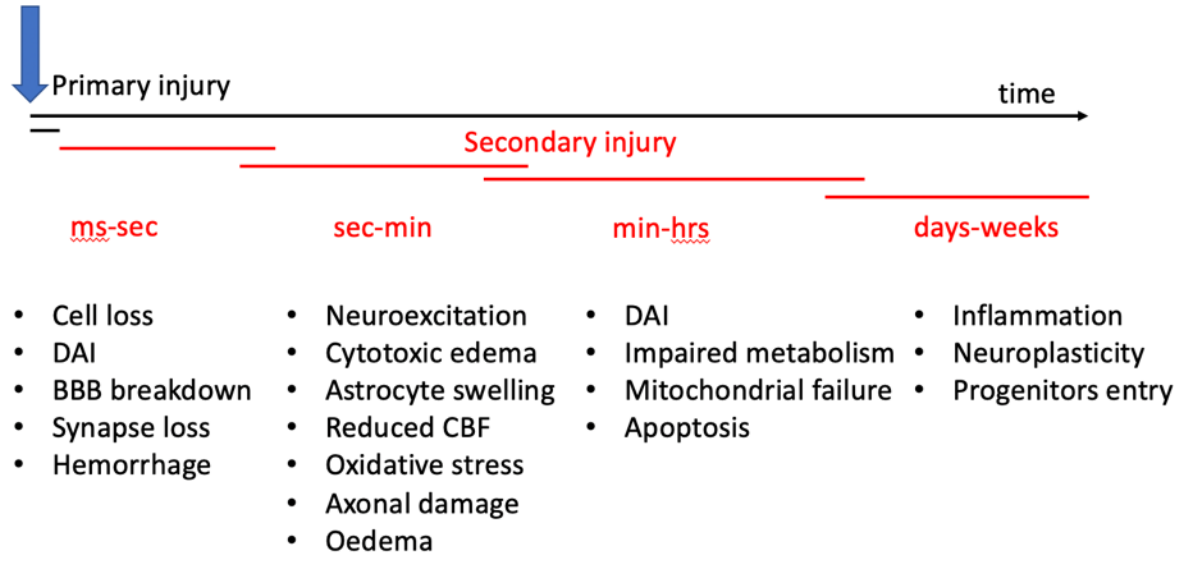

Figure 1.1. Primary and secondary injury mechanisms.

Schematic representation of post-injury mechanisms activated after an initial traumatic injury to the brain (Adapter from Gupta and Przekwas, 2013)

Pathological and behavioural changes after TBI are likely to be heterogeneous, and influenced by many factors. Cellular and sub-cellular changes could both contribute to brain pathology. Factors that could potentially affect these changes, as well as recovery process after mTBI, include the age at injury, sex, as well as the number of injuries and interval between injuries. Compelling studies have found aged adults are particularly vulnerable to TBI with increased cognitive and behavioural dysfunction, as well as higher morbidity and mortality rates (Pannese, 2011, Popa-Wagner et al., 2011). The underlying mechanisms causing this may be associated with the properties of the aged brain itself, including decreased brain plasticity (Sapoznik et al., 2006), a shift from balanced homeostatic inflammatory state to a pro-inflammatory state (Popa-Wagner et al., 2011), as well as increased cellular and subcellular dysfunction (Timaru-Kast et al., 2015). In children, injury to the brain may be viewed as more complex as the brain is still developing. Historically, the brain of a child was considered to have a higher capacity for plasticity, which was thought to favor recovery from brain injury (Johnston et al., 2009, Kolb and Gibb, 2011). However, with regard to mTBI, there are a proportion children that present with delayed deficits despite an apparent return to normality acutely after injury (Karver et al., 2012, Eisenberg et al., 2014). In addition, it has been observed that many children do not fully recover and have persisting PPCS weeks, months,

and even years after injury (Anderson et al., 2017, Keenan et al., 2018). Therefore, whether the young are protected from or vulnerable to mTBI is still controversial. Similarly, the literature is mixed with regard to explaining how sex influences the cellular reactions after TBI. Clinical studies of mild and moderate brain injuries among female general patients and sports injuries have found that injuries in females resulted in significantly worse outcomes, those include higher somatic and motor symptoms, increased difficulties with memory and cognition, and higher levels of depression at 6 months post mild to moderate injury, as well as higher drowsiness and sensitivity to noise at 1 year after mild injury (Dick, 2009, Preiss-Farzanegan et al., 2009, Bazarian et al., 2010, Frommer et al., 2011, Laker, 2011, Covassin and Bay, 2012). However, the major experimental studies report female animals with better recovery than male animals after brain injury (Gupte et al., 2019). The number of injuries, and interval between them are another risk factor for mTBI. Animal studies modeling rmTBI using various number of injuries and interval between injuries, suggested short-time interval between injuries exacerbated injury pathology (Grant et al., 2018, Mouzon et al., 2018a), while others found the interval between injuries did not have any additional effects (Fujita et al., 2012, Hall et al., 2016). The complex and inconclusive clinical and/or experimental findings on how these risk factors influence the pathological changes and recovery process after mTBI suggest that more studies are warranted.

1.1. MILD TRAUMATIC BRAIN INJURY

Even though there is no overt brain damage following mTBI, the initial physical stretch and stress to the brain can potentially initiate a pathophysiological cascade in the brain, in which neuroinflammation, brain edema, hypometabolism, and vascular injuries occur (Blennow et al., 2012, Jordan, 2013). Additionally, behavior and motor function deficits, as well as cognitive impairments have been reported at both short-term and long-term after mTBI (Kraus et al., 2007, Tay et al., 2010). These deficits after mTBI may be caused by cellular, sub-cellular, and molecular reactions, which are still under active investigation and are yet to be fully elucidated.

1.1.1. The neuronal reaction to mTBI

Neurons have a distinct morphology with a soma, an axon and dendrites. At its simplest, the axon transmits signal from one neuron to the terminals of another neuron, where it forms synapses with neuronal connections and information is converted. Dendrites receive connections from other neurons which act to propagate the electrochemical stimulation on the neuronal surface through synapses (Brodal, 2004). The synapse consists of a pre-synapse, synaptic cleft and post-synapse. Synaptophysin and post synaptic density protein 95 (PSD-

95) can be used as presynaptic and postsynaptic markers, respectively. Synaptophysin is a calcium binding glycoprotein (38,000KD), and is an integral membrane protein localized to synaptic vesicles (Wiedenmann et al., 1986). PSD-95, a member of the membrane associated guanylate kinase family, accumulates at the post synaptic density of neurons which binds excitatory receptors (Hunt et al., 1996). Spines are the dendritic membranous protrusions used for synaptic strength and electrical transmission. There are hundreds to thousands of spines on a neuron. They can be classified into three categories: mushroom, stubby and thin spines according to their shapes and lengths (Fig 1.2) (Harris et al., 1992). Mushroom spines have a large head but a small neck and are usually regarded as mature spines (Harris et al., 1992), whereas thin spines are long and fine and are regarded as immature and unstable spines (Petrak et al., 2005). Stubby spines are usually regarded as the transitional stage between mushroom and thin spines. Spines are an important formation of synapses, of which the plasticity including long-term potentiation and depression, are essential for learning and memory. The proportion of mature/immature spines is vital for synaptic signaling and alterations in dendritic spine density have been inversely linked to cognitive function (Penzes et al., 2011). In the context of moderate and severe TBI, neuronal degeneration and death, dendritic beading and fragmentation, as well as spine and synapse reduction have been observed not only at the injury site, but also in remote areas distal to the injury site including hippocampus and contralateral cortex (Gao et al., 2011, Winston et al., 2013, Zhao et al., 2015, Zhao et al., 2016). With regard to mTBI, one report states that the injury did not cause overt neuron death and left the cortex intact without cavity, however, it was found the injury caused dendritic beading in the peri-injury cortex at early as 2hrs post-injury (Sword et al., 2013). It was also observed that mild injury induced dendrite and spine degeneration, as well as an decrease in the number of synapses in the injured cortex at 3 days post-injury (Gao and Chen, 2011), additionally, at the same time point after mild injury, a significant decrease in dendritic density, as well as dendritic branching were reported in the parietal cortex (Ratliff et al., 2020). Injury related dendritic damage may extend beyond the injured area, at 3 days post injury, a reduction in dendritic density as well as dendritic branching were observed in the hippocampus (Ratliff et al., 2020). At 32 days after mild injury, decrease in spine density was observed in the layer 2/3 pyramidal neurons of the infralimbic region (Zhao et al., 2018).

Figure 1.2 Dendritic spines classification.

Dendritic spines are grouped into three classes: thin, mushroom, and stubby spines according to their shape and appearance (Adapted from Rodriguez et al., 2008)

Axons are long projections, which are used to conduct electrical impulses and are vital for neuronal transmission. The neuronal cytoskeleton, comprising microtubules, actin filaments and neurofilaments (NFs), plays an important role in the establishment, remolding and maintenance of neuronal architecture, as well as the morphology and integrity of axons (Conde and Cáceres, 2009, Kapitein and Hoogenraad, 2011). NFs play an important role in controlling axonal diameter and conductance (Yuan et al., 2012), while microtubules and actin filaments are mainly involved in axonal specification and growth, as well as long- and short-distance active axonal transport (Sainath and Gallo, 2015). Axons are susceptible to brain injury with long axonal projections in the white matter tracts, and the unmyelinated axon in the brain is particularly vulnerable (Johnson et al., 2013b, Smith et al., 2013a, Wong et al., 2017). TBI can cause damage to axons throughout the white matter, which is identified as diffuse axonal injury (DAI). It is a pathological hallmark of TBI (Frati et al., 2017), and has been widely observed in moderate and severe TBIs (Vieira et al., 2016, Rabinowitz et al., 2018). DAI encompasses a spectrum of primary mechanical breaking of the axonal cytoskeleton to transport interruption, through secondary physiological changes. Additionally, it is characterized by axonal swellings or varicosities, large terminal bulb formations, and may finally lead to secondary axotomy and axonal disconnection (Smith et al., 2003, Johnson et al., 2013b). Immunohistochemical identification of Amyloid Precursor Protein (APP) accumulation in damaged axons is the gold-standard neuropathological approach for the diagnosis of DAI post-mortem (Sherriff et al., 1994, Johnson et al., 2013b). In severe TBI, the initial injury force may induce immediate axonal disconnection, however, this is rare in mTBI (Johnson et al., 2013b). With regard to mTBI, axonal swelling was observed dispersed across the neocortex at 2 days after injury in mice (Vascak et al., 2017). Moreover, significantly higher βAPP positive axons within the corpus callosum was observed at 2 hrs and 48 hrs after injury

in mice (Henninger et al., 2016). At 1 week post injury, degenerating axon terminals detected by silver staining were observed within the corpus callosum, entorhinal cortex, cerebra peduncle and fornix in mice (Chen et al., 2018). Likewise, higher axonal degeneration in the external capsule and internal capsule was also observed at 7 days post injury in mice (Chuckowree et al., 2018). At longer timepoint, electron microscopy revealed degenerating axons among intact fibers in the corpus callosum at 6 weeks post injury in mice (Mierzwa et al., 2015). These axonal changes have been suggested to correlate with functional, cognitive and emotional impairments (Hellstrøm et al., 2017). Therefore, with regard to mTBI, it has been proposed that the primary stretching force disrupts axonal membranes, and the secondary metabolic and ionic dysfunction may then affect the axonal cytoskeleton, disrupt axonal transport, and form swellings in contiguous axons, which finally causes axonal disconnection (Johnson et al., 2016). Axonal myelin may also be disrupted and degraded after TBI, which may trigger and exacerbate neuroinflammation as the debris may be stimulating microglial activation (Vargas and Barres, 2007, Clarner et al., 2012).

1.1.2. The astrocyte reaction to mTBI

Astrocytes are star-shaped glial cells in the brain. They contain a density of intermediate filaments, such as glial fibrillary acidic protein (GFAP) which has been used as a marker for astrocyte activation. GFAP is markedly up-regulated after TBI and stroke (Liu et al., 2014, Yu et al., 2016), additionally, levels of GFAP are suggested to be correlated with the severity of TBI and could be used as a predictor for outcomes after TBI (Luoto et al., 2017, Hossain et al., 2019). In healthy brains, astrocytes play vital roles in neural circuit function by maintaining the homeostasis of water, ions, blood flow and neurotransmitters (Sofroniew and Vinters, 2010, Pekny and Pekna, 2014). Additionally, they are the pivotal responders to all forms of brain insults. After a TBI, astrocytes become activated, a process referred to as reactive astrogliosis. These activated astrocytes may be beneficial by protecting neural tissue through isolating the lesion, and restraining the damage (Hamlin et al., 2001). Additionally, they are involved in inflammation and blood brain barrier (BBB) regulation, as well as synapse formation and function (Burda et al., 2016). However, astrocytes may become detrimental by producing pro-inflammatory cytokines and chemokines, which could cause further damage to the brain (Burda et al., 2016). Astrocyte activation could be long-lasting with activated astrocytes in the cortex and hippocampus 6 months after TBI reported (Mouzon et al., 2014). In the context of mTBI, reactive astrogliosis and hypertrophic astrocytes have also been observed in various regions, including the injured cortex, corpus callosum, and hippocampus after acute and chronic injuries (Goodrich et al., 2016, Rodriguez-Grande et al., 2018), which could partially contribute to post-TBI dysfunction (Burda et al., 2016). Excitotoxicity is a

common component of secondary brain injury after mTBI (Mayeux et al., 2017, Shokouhi et al., 2020), which could be detrimental to neurons. Astrocytes provide functional support for neurons by recycling of the glutamate from synapses, thus to prevent the accumulation of extracellular glutamate, in which the glutamate transporters, such as EAAT1 and EAAT2 are required (Chen and Swanson, 2003).

1.1.3. The microglia reaction to mTBI

Microglia are myeloid-derived cells (Ransohoff and Perry, 2009), which are a type of neuroglia located in the brain and spinal cords. Microglia are involved in synaptic pruning, central nervous system (CNS) repair and immune defense. Ionized calcium binding adaptor molecule 1 (Iba1) is a protein whose expression is specific to microglia/macrophages (Imai et al., 1996). Although its expresssion is thought to increase with microlial activation, it is considered as a marker of all microglia, rather than an acitivated subset (Walker and Lue, 2015). Iba1 expression is upregulated in activated microglia following TBI (Gao et al., 2015, Woodcock and Morganti-Kossmann, 2015). In response to TBI, microglia can migrate to the injury site, build a protective environment and alleviate the deleterious sequelae of brain injury (Faden et al., 2016). The acute function of microglia involves removing the cellular and molecular debris, in order to restore the normal environment of the brain (Faden et al., 2016, Donat et al., 2017). However, microglia can also become dysregulated, produce high levels of pro-inflammatory, noxious substances and cytotoxic mediators, that contribute to neuronal dysfunction, cell death, and affect synaptic plasticity and cognition (Loane and Kumar, 2016). Microglial activation could be long-lasting with observations of activated microglia within the injured cortex and hippocampus at 6 months and even up to 1 year after brain injury (Mouzon et al., 2014, Norden et al., 2015). In the context of mild TBI, microglial reactivity has also been reported in the cortex, corpus callosum and hippocampus after acute and chronic injury (Lafrenaye et al., 2015, Goodrich et al., 2016).

1.2. AGE AT INJURY AS A RISK FACTOR FOR mTBI

The elderly population is growing worldwide, compelling studies have revealed that the ageing process is associated with increased CNS vulnerability evidenced by an increasing rate of cognitive decline, neurological dysfunctions, mortality and morbidity (Pannese, 2011, Popa-Wagner et al., 2011). The mechanism underlying this vulnerability may be relevant to the brain changes during ageing. These changes include brain atrophy (Pannese, 2011), a reduction in brain plasticity (Petralia et al., 2014, Seib and Martin-Villalba, 2015), a pro-inflammatory state (Popa-Wagner et al., 2011), higher oxidative stress due to increased oxidative substance

production and decreased antioxidative generation (Romano et al., 2010), as well as metabolism disruption with decreased glucose uptake and increased mitochondrial dysfunction (Craft, 2005). Clinical data strongly suggests elderly individuals account for a disproportionately high incidence of TBI that is associated with poorer outcomes, with the oldest having the poorest outcomes after TBI, along with slower rehabilitation(Cheng et al., 2014b, Seagly et al., 2018), which causes them fall behind younger patients (Mosenthal et al., 2004). The potential underlying mechanisms may include less compensatory neurons and higher systemic complications suggested by clinical findings (Davis et al., 2011), as well as higher oxidative damage and higher mitochondrial dysfunction suggested by experimental studies (Gilmer et al., 2010, Itoh et al., 2013).

Children's brains are still undergoing development, with brain connections being formed and pruned, in order to gain new functions and shape the brain. TBI in children was previously thought to be offset by their adaptive properties, among which, plasticity is one of the most predominant adaptive factors. Plasticity enables the brain to adapt to brain injury through modulating genetic, molecular and cellular mechanisms, and then affect synaptic connections and neural circuitry formation (Johnston, 2004, Lau and Zukin, 2007), which may increase impact tolerance as well as benefit the recovery process (Chen et al., 2015). This plasticity is suggested by abundant neural stem cells, quicker toxic protein (such as amyloid beta) removal, and quicker metabolism recovery in young brains (Venkitaramani et al., 2007, Deng-Bryant et al., 2011, Zhang et al., 2017b). In the context of mTBI, some children may fully recover within the first few days or weeks post injury, however, a proportion of children do not recover and develop post-concussive symptoms which may persist even years after injury (Anderson et al., 2017, Keenan et al., 2018). These long-lasting and later onset behavioural and cognitive dysfunctions following mTBI were also supported by animal studies (Mychasiuk et al., 2015a, White et al., 2017). Therefore, the recovery trajectory after mTBI in children is complex and cannot be solely explained by plasticity or vulnerability.

1.2.1. The effect of age on the neuronal reaction to TBI

During normal ageing, the loss of neurons is initially observed in the cortex and hippocampus (Peters, 2002), furthermore, the size of neurons has been found to decrease with ageing, which suggests reduced arborization of axons and dendrites, and a subsequent reduction in synapses (Hof and Morrison, 2004). With the emergence of advanced imaging techniques, more studies suggest that there is not a substantial reduction in neurons in most brain regions (Egawa et al., 2016). In the context of TBI, increased lesion size and neurodegeneration in

the hippocampus and thalamus were observed in aged rather than young mice at 7 days after moderate TBI (Kumar et al., 2013).

Synaptic dysfunction may contribute to the cognitive deficits in the aged. Decreased synaptic density in the cortex and hippocampus has been observed with aging and is associated with cortical dysfunction and cognitive decline (Morrison and Baxter, 2012). Additionally, synaptic functional changes can be attributed to reduced glutamate release, and altered postsynaptic structure (including dendritic spine density decrease and synaptic contact area reduction) (Morrison and Baxter, 2012). With ageing, pyramidal neurons have been shown to exhibit progressive regression in dendritic arbors in the cortex (Kabaso et al., 2009), as well as reduction in complexity of apical and basal dendrites has also been observed with ageing (Kabaso et al., 2009). Spine loss has also been found in pyramidal neurons (Cupp and Uemura, 1980), this is further supported by a decrease in the density of apical and basal dendritic spines with ageing (Kabaso et al., 2009). Dendritic and spine changes with ageing could potentially affect the functional properties of individual neurons, and subsequent cognitive and behavioural functions.

In the context of TBI, the apical dendritic branches, total dendritic length, and spine density in the nucleus accumbens decreased after chronic moderate TBI in adolescent mice, which was not observed in adult mice (Cannella et al., 2019). However, at 3 months after injury, N-methyl-d-aspartate receptor subtype 2B (NR2B) expression in the hippocampus was decreased by 20% in both adolescent and adult mice, and there was no difference in NR2B expression in the cortex between injured and sham adolescent or adult mice (Mannix et al., 2017).

White matter impairment has been associated with behavioural and cognitive decline (Farokhian et al., 2017). It has been demonstrated that there is a decrease in brain white matter but not in grey matter in aged patients (Joo et al., 2010). Additionally, white matter volume reduction in the orbitofrontal cortex and corpus callosum has been observed with ageing (Bartzokis et al., 2003, Hedden and Gabrieli, 2004). Reduction in myelinated axons and mild demyelination in the subcortical white matter has also been reported, which may contribute to the functional decline seen in normal ageing (Xie et al., 2016). Furthermore, a decrease in the number of optic nerve axons and degeneration of axons were observed with ageing (Stahon et al., 2016). This thicker myelin also had longer and thicker mitochondria, which increased oxidative stress and disrupted ionic homeostasis, increased nodal and paranodal distance and disrupted axonal function (Stahon et al., 2016). Additionally, axonal transport including mitochondrial transit has been observed to decline during ageing (Milde et al., 2015). Axonal regenerative capacity has also been found to decrease with ageing

(Roozbehi et al., 2015). Moreover, higher diffuse axonal injuries, vascular anomalies, and behavioural deficits have been observed in aged patients (Gattu et al., 2016).

In the context of TBI, white matter area has been shown to be decreased in adult but not in adolescent mice compared to sham mice after rmTBI (Mannix et al., 2017). Additionally, axonal injury in the corpus callosum, detected by APP immunoreactivity, was greater in aged, as opposed to young mice, observed after acute and chronic phases after rmTBI (Ferguson et al., 2017b, Mouzon et al., 2018b). However, the neurofilament response in the optic tract was robust in young injured mice but markedly attenuated in old injured mice after mTBI (Cheng et al., 2018). Thus, how the brain injury interacts with age at injury is still inconclusive.

Glymphatic dysfunction has been shown in animal model of TBI (Christensen et al., 2020), which may contribute to the build up of proteinaceous waste products, including p-tau and beta-amyloid in TBI patients (Jessen et al., 2015). Additionally, during ageing, glymphatic system function decreases dramatically (Jessen et al., 2015). Thus, the accumulated effects of injury and ageing may exacerbate the development of tau pathology and neuronal dysfunction (Iliff et al., 2014).

1.2.2. The effect of age on the astrocytic reaction to TBI

The phenotype of astrocytes changes between young and aged patients, from long and slender processes in young patients to short and stubby processes in older subjects (Jyothi et al., 2015). This inflammatory morphological change has also been observed in rodents, and was region dependent, increased in striatum but reduced in retina (Jinno, 2011). In normal ageing, increased astrocyte activation with exacerbated pro-inflammatory gene expression has been observed, which could disrupt BBB function (Wu et al., 2005, Clarke et al., 2018), and increase cytokine production to exacerbate the inflammatory response (Clarke et al., 2018). Additionally, astrocyte activation could also accumulate and increase reactive oxidative stress (Ishii et al., 2017), and interrupt mitochondrial function (Sundar Boyalla et al., 2011). Astrocytes have also been suggested to interact with microglia to increase pro-inflammatory cytokine expression during ageing (Sierra et al., 2007).

In the context of TBI, an increased astrogliosis response in the optic nerve was observed at 1 day after rmTBI in aged compared to young mice (Tzekov et al., 2016). Additionally, reactive astrogliosis in the CC was observed in both young and aged mice at 15 days post injury, and the average percentage of area of astroglial activation for aged mice was higher compared to young mice for female in the CC at 15 days post-injury (Ferguson et al., 2017b). At 1 day post-

injury, the average percent area of GFAP for the injury group was greater compared to the sham group for female and male mice. Aged mice showed higher astrogliosis compared to young mice in the CC with regard to GFAP staining (Mouzon et al., 2018b).

1.2.3. The effect of age on the microglial reaction to TBI

During the ageing process, the number, location and the morphology of microglia changes, which may also be associated with changes in microglial function. Increased microglial activation has been observed during normal ageing (Grabert et al., 2016), and additionally, it has been observed that the population of microglia increases with ageing (Mouton et al., 2002). In aged brains, microglia are shorter and less-branched (Hwang et al., 2008). Furthermore, microglia showed decreased ramified structure during ageing, which could potentially affect their normal function (Wong, 2013), including toxicity removal (Floden and Combs, 2011). In aged animals, microglia undergo a priming process, which is associated with higher inflammation, such as increased expression of inflammatory markers including major histocompatibility complex 2 (Henry et al., 2009).

In the context of TBI, a stronger microgliosis response in the corpus callosum was observed in young compared to old mice at 1 day after rmTBI (Mouzon et al., 2018b). However, at 7 days after injury, there were highly reactive microglial phenotypes in the injured cortex, hippocampus, and thalamus in aged rather than young mice after moderate TBI (Kumar et al., 2013). Furthermore, at 15 days after mTBI, the injured mice showed a notable increase in Iba1 immunoreactivity in the CC, and the average percent area of Iba1 immunoreactivity was higher for aged compared to young males (Ferguson et al., 2017b). Even though the microglial activation at 2 days post-injury was not different between young and aged brain injured mice, significantly higher microglial activation was observed in the optic tract of aged compared to young brain injured mice at 7 days after repetitive brain injury (Cheng et al., 2018). When observed at longer timepoints (3 months), microglial reactivity in the CA1 area did not differ between adolescent and adult mice after rmTBI (Mannix et al., 2017).

To sum up the influence of age at injury on outcomes following mTBI. The aged brain is characterized with brain atrophy, axonal degeneration, a higher inflammation state, and compromised neuronal connections. On the contrary, the young brain is still undergoing development and maturation, suggesting the young brain is characterized with resilience and plasticity. Thus, it suggests the aged brain is more susceptible than the young brain to assault (Pannese, 2011, Popa-Wagner et al., 2011). With regard to mTBI, however, compelling

studies have observed that a proportion of children with mTBI showed vulnerability with delayed and prolonged post-injury symptoms (Donders and Warschausky, 2007, Lichte et al., 2015). In order to identify the influence of the age at injury on outcomes following mTBI, various studies have investigated the cellular responses after mTBI at different time points in the lifespan. However, to-date, studies have not comparatively assessed in a single species, the effects of mTBIs received at different timepoints across the entire lifespan. Since age plays a role in the response to mTBI, it is therefore necessary to fully understand the differences in cellular reactions across the entire lifespan.

1.3. SEX AS A RISK FACTOR FOR mTBI OUTCOME

Sex hormones influence multiple cellular functions including inflammation, oxidative stress, excitotoxicity and mitochondrial function (Demarest and McCarthy, 2015, Gunther et al., 2015, Villapol et al., 2017). The majority of TBI studies have been conducted in the male population, as they were thought to be more likely to sustain TBIs than females during their lifetime (Rickels et al., 2010), however, it has been recently observed that females in sex-comparable sports had a higher incidence of reporting concussion than males (Covassin et al., 2016). Additionally, it has been observed that most moderate to severe brain injuries occur in males and result from occupations, such as professional contact sports, construction or military occupations, while most mild injuries occuring in females result from domestic violence (Chang et al., 2014, Colantonio, 2016).

Sex is one of the most controversial risks factor for TBI. Mixed and complicated outcomes between different sexes have been observed after brain injury. While some clinical studies found females have unfavorable outcomes compared to males, other patient studies did not find any difference in cognitive symptoms between sex (Bazarian et al., 2010, Covassin and Bay, 2012). In the clinic, observational studies have reported that outcomes are generally poorer in female patients compared to males after moderate and severe TBI (Kirkness et al., 2004, Morrison et al., 2004). In addition to this, higher severity of acute post-injury symptoms has been reported in females compared to males patients (Berz et al., 2013). A worse working memory outcome was found in female compared to male patients at 10 weeks after mild injury (Hsu et al., 2015). Furthermore, even at 3 years after mTBI, higher frequency of PPCS and disability was observed in female compared to male patients (Styrke et al., 2013). However, females with mTBI have also been reported without impairments on neuropsychological performance, which may indicate female sex plays a protective role (Bai et al., 2019). This is supported by other studies that reported females have superior executive functioning, verbal memory and learning performance, when compared with males after acute TBI (Niemeier et

al., 2014) and less possibility of persisting PPCS after chronic TBI (Von Der Heide et al., 2013). Conflicting results also exist within experimental studies. Worse outcomes in females than males have also been found with regard to mTBI (Ferguson et al., 2017b), however, compelling experimental studies showed better behavioural outcomes (better balance and coordination) (Mychasiuk et al., 2016), and better cognitive functions (better performance in learning and in the Barnes Maze) in female than male mice (Velosky et al., 2017).

The presence of neuroanatomical sex differences has been investigated in animal research. It has been reported that there was no differences in neocortical thickness (Stewart and Kolb, 1988), or neuronal soma size or density in the primary visual cortex between male and female rats (Reid and Juraska, 1992). In contrast, a larger cortical volume was reported in male compared to female rats (Reid and Juraska, 1992). Additionaly, thinner cortices were reported in female compared to male mice (Velosky et al., 2017). In the context of TBI, less neuronal loss or degeneration (Armstead et al., 2016, Free et al., 2017), less BBB breakage and brain edema (O'Connor et al., 2006) and lower cerebral blood flood (Armstead et al., 2016) were reported in female compared to male animals after injury.

Sex differences also exist with regard to the density of dendritic spines. It has been reported in experimental studies that females have less dendritic branching in the prefrontal cortex than males, whereas, in the agranular insular cortex, females had greater apical arbors than males (Kolb and Stewart, 1991). Spine density is sexually dimorphic, with male rats exhibiting greater spine density than females on principal cell densities, which possibly indicated more excitatory inputs from any number of afferent populations (Rubinow et al., 2009). In the context of TBI, female mice showed less reduction in dendritic complexity of pyramidal neurons in the ipsilateral prefrontal cortex compared to male injured mice (Semple et al., 2017). Additionally, it is also reported that female rats with less density of spines in the nucleus accumbens compared to male rats, but both experienced a significant reduction following the mTBI (Hehar et al., 2015).

Sex differences in the diameter and g ratio (the ratio of the inner axonal diameter to the total outer diameter) of myelinated axons have been reported, with thicker axonal diameters and higher g ratios in female rats compared to in male rats (Pesaresi et al., 2015). Moreover, a significant sex difference in the ratio of unmyelinated to myelinated axons has been reported, with females having higher numbers of unmyelinated axons and a greater ratio of unmyelinated to myelinated axons in the corpus callosum. No differences in the size of either axon type or myelin thickness was observed. These results suggested that there was a sex difference in the proportion of axon types (Mack et al., 1995). In the context of TBI, a sex

dependent difference in axonal pathology has been shown, with reduced phosphorylated tau in the cortex and hippocampus in aged female but not in aged male injured mice (Ferguson et al., 2017b). This difference was not observed in young mice. Among younger mice, the effect of sex on white matter degeneration was similar among females and males after injury (Ferguson et al., 2017b).

The activation of astrocytes was suggested to occur in a sex dependent manner. Under basal unstimulated conditions, astrocytes from male and female mice showed similar mRNA levels of anti and pro inflammatory cytokines (Santos-Galindo et al., 2011). However, sex differences in the distribution of GFAP immunoreactivity were detected in rat hippocampus and globus pallidus (Garcia-Segura et al., 1988). Furthermore, it was reported that the immunoreactivity of GFAP in CA1 and CA3 areas was significantly higher in male compared to age-matched female rats (Conejo et al., 2005).

In the context of TBI, it is reported that injury induced astrogliosis in the peri-injury area was comparable between female and male rats at 1 day after brain injury (Gunther et al., 2015). Furthermore, another study reported GFAP mRNA levels without any sex related differences in the rat prefrontal cortex and hippocampal at around 17 days after final injury (Yamakawa et al., 2017). At 32 days after the repetitive injury, increased astrocyte reactivity in the corpus callosum and optic tract in the ipsilateral side was similar between female and male injured mice, however, in the optic tract in the contralateral side, male injured mice had significantly increased GFAP immunoreactivity than female injured mice (Velosky et al., 2017).

Sex differences have been reported for the activation of microglia. No sex difference in glial density in the primary visual cortex was observed in rats under basal unstimulated conditions (Reid and Juraska, 1992). However, sex dependent microglia gene expression was reported, with major genes expressed in male mice belonging to the inflammatory process including regulation of cell migration and cytokine production, while most genes expressed in female mice were associated with inflammatory response inhibition and repair promotion (Villa et al., 2018). Furthermore, it was also reported that microglia in male mice had increased phagocytic activity and higher ROS levels in the brain, whereas microglia in female mice had increased production of TNF-α and IL-1β (Doran et al., 2019).

In the context of TBI, it was reported that there were no sex dependent differences in phagocytic activity in resident microglia in brain injured mice at either 1, 3 or 7 days after injury (Doran et al., 2019). However, male mice showed a significant influx of peripheral myeloid cells by 1 day post-injury followed by proliferation of resident microglia at 3 days post-injury.

In contrast, myeloid infiltration and microglial activation responses in female injured mice were significantly reduced (Doran et al., 2019). Additionally, at 17 days after the final injury, there was an increase in the number of Iba1 positive microglia in the ventromedial hypothalamus in male, but not in female rats (Yamakawa et al., 2017).

In summary, sex dimorphs exist in various cellular changes, including the cortical thickness, spine density, axonal degeneration, and glial response. Conflicted results have been found with regard to how sex affects outcomes following TBI. Despite differences being identified in the cellular response to TBI between sexes, the results are inconclusive.

1.4. REPETITION OF INJURY AS A RISK FACTOR FOR mTBI OUTCOME

Contact sport (like football, boxing, ice hockey, martial arts and soccer) athletes and military personnel are at high risk of suffering repetitive mTBI (Gardner and Yaffe, 2015). The initial injury may recover within a few days or weeks, however, the effects of additional injuries could potentially result in long-term neurological and functional deficits (Smith et al., 2013b). Clinical studies found rmTBI was accompanied with prolonged depression, reduced cognitive performance and prolonged recovery (Ling et al., 2017, Merritt et al., 2018, Maynard et al., 2019). Furthermore, it has also been associated with a variety of neurodegenerative disorder related pathologies, including pathologies in amyloid beta, tau, TAR DNA-binding protein 43 (TDP-43), and chronic traumatic encephalopathy (Saing et al., 2012, Solomon and Zuckerman, 2015, Xu et al., 2016). Additionally, chronic neuroinflammation, ongoing axonal degeneration and white matter atrophy, cerebrovascular abnormality, depositions of abnormal tau and neurobehavioural impairments following rmTBI were also observed in animal studies (Mouzon et al., 2014, Petraglia et al., 2014, Ojo et al., 2016). In order to understand the pathology of rmTBI, numerous animal models have been used to model the changes after injury. Studies using these models have been associated with inconsistent pathological and functional findings, which may be partly attributed to the variable number of injuries and intervals between them (Meehan III et al., 2012). Various injury parameters, including number of injuries and interval between injuries, were applied to model rmTBI, with some suggesting shorter interval between injuries was associated with exacerbated pathology (Weil et al., 2014, Gold et al., 2018), while other suggesting the interval between injuries do not affect long-term pathological and behavioural outcomes (Fujita et al., 2012, Hall et al., 2016).

Studies modeling repetitive mild injuries on neuronal changes report inconsistent outcomes. There are studies reporting no overt neuronal death or cortical damage after repetitive injury. Boltman and colleagues report no significant neuronal degeneration in the entorhinal cortex

at 9 days following 5 injuries at 2 day intervals (Bolton and Saatman, 2014). At 14 days after injury, it has been observed that there was no overt structural change or neuronal loss after 4 injuries at 7 day intervals (Xu et al., 2016). Furthermore, at later time points, no overt structural changes or neuronal loss has been observed at 40 days after 2 injuries at 3 day apart (Weil et al., 2014). Moreover, no neuronal loss in the injured cortex following 5 injuries at 2 day intervals has been observed at 2 months after injury (Gold et al., 2018). It has been reported that the second mild injury delivered at a 1 day interval did not cause cell death in the cortex 7 days after the initial injury (Shitaka et al., 2011). However, 10 injuries at 1 day intervals significantly decreased the number of neurons in the injured cortex at 2 months after injury (Gold et al., 2018).

Diffuse axonal injury is considered to be a key feature of mTBI pathology (Bigler, 2013). Studies modeling repetitive mild injuries on axonal changes found higher axonal damage after repeat brain injuries. After acute brain injury, there was increased axonal injury in the external capsule, at 5 days after 5 injuries at 1 day intervals and 9 days after 5 injuries at 2 day intervals (Bolton and Saatman, 2014). At 7 days after brain injury, increased APP was observed in the cortex, corpus callosum, and external capsule after 2 injuries at 1 day intervals (Shitaka et al., 2011). In this study, dystrophic myelinated axons, axoplasmic collapses, and cytoskeletal disruptions were also observed (Shitaka et al., 2011). At 14 days after brain injury, higher axonal degeneration in the corpus callosum was observed after 4 injuries at 1 day intervals (Hylin et al., 2013), and additionally, increased axonal degeneration profiles were also reported in the corpus callosum at 14 days after 4 brain injuries at 7 day intervals (Xu et al., 2016). At 40 days after brain injury, axonal degeneration was significant and widespread. It was particularly pronounced in the corpus callosum, caudate putamen and dentate gyrus after 2 injuries at 3 day intervals but not at 20 day intervals (Weil et al., 2014). After chronic brain injury, white matter abnormalities and corpus callosum atrophy developed at 2 and 6 months after 5 injuries at 2 day intervals and 10 injuries at 2 day intervals, respectively (Gold et al., 2018). Furthermore, at 6 months and 12 months after brain injury, the average thickness, as well as increased APP immunoreactivity of the corpus callosum was reduced after 5 injuries at 2 day intervals (Mouzon et al., 2014). Furthermore, increased silver staining in the external capsule was observed at 8 days and 22 days after 2 brain injuries at 1 day intervals, but not at 92 days post-injury (Fidan et al., 2016). In summary, increased axonal pathological changes have been widely observed at both short-term and long-term timepoints after repetitive injuries.

Understanding of microglia changes after repetitive mild injuries is incomplete. After acute injury, at 5 days after injury, there was no differences in microgliosis in the hippocampus but

it was higher in the entorhinal cortex following 5 injuries at 1 day intervals (Bolton and Saatman, 2014). At 7 days post-injury, there was higher microglial activation in the ipsilateral cortex, hippocampus, and thalamus, and corpus callosum following 2 injuries at 1 day intervals (Shitaka et al., 2011). However, at 8 days after injury, there was no significant microglial activation in cortex, hippocampus or amygdala after 2 injuries at 1 day intervals (Fidan et al., 2016). At 14 days post-injury, higher microgliosis was found in the corpus callosum after 4 injuries at 1 day intervals (Hylin et al., 2013). At 40 days post injury, microglial activation was evident in the sensorimotor cortex, hippocampus, and thalamus after 2 injuries at 3 day intervals (Weil et al., 2014). At 12 months after chronic injury, there was no differences in microglial activation in the cortex or corpus callosum after 5 injuries at 2 day intervals (Mouzon et al., 2014).

Similarly, studies on astrocyte response after repetitive mild injuries are mixed. After acute injury, there was no differences in astrocytosis in the entorhinal cortex, but it was higher in the hippocampus at 5 days following 5 injuries at 1 day intervals, and at 9 days following 5 injuries at 2 day intervals (Bolton and Saatman, 2014). Additionally, higher astrogliosis in the corpus callosum at 14 days after 4 injuries at 1 day intervals was observed (Hylin et al., 2013). Moreover, evidence of astrocytic activation was observed in the sensorimotor cortex, hippocampus, and thalamus at 40 days after 2 injuries at 3 day intervals (Weil et al., 2014). After chronic injury, there was also higher astrogliosis in the somatosensory cortex at 12 months after 5 injuries at 2 day intervals (Mouzon et al., 2014). Thus, increased astrocyte activation has been reported at longer timepoints after repetitive injuries.

Although a number of animal studies modeling rmTB have been performed, complex and conflicting cellular and subcellular responses have been found, which may be partially attributed to the various number of injuries and injury time intervals used. Therefore, more animal studies on how the number of injuries and time intervals influence mTBI outcomes are needed.

The Controlled Cortical Injury (CCI), Weight Drop Injury, Blast Injury and Fluid Percussion Injury (FPI) models have all been used to replicate aspects of mTBI, each having their own strengthens and weaknesses. The CCI and FPI models have the best characterization over varied injury levels. These two models also lend themselves more easily to repetitive injury paradigms, which may also be particularly important when studying mTBI (Laurer et al., 2001, Kane et al., 2012, Wang et al., 2012). CCI is a well-characterized model of focal TBI. The major disadvantage of CCI models is the need to perform craniotomies, which in itself disrupts

the normal parenchyma, activates immune cells/microglia, and can trigger of gliosis in the brain (Lagraoui et al., 2015).

The fluid percussion injury can be used to deliver both midline and lateral fluid percussion injuries (Dixon et al., 1987, McIntosh et al., 1987, Lyeth et al., 1990). This model is widely used as it mimics clinical contusion without skull fracture (Cernak, 2005). The advantages of the fluid percussion model include that it is a widely used model with well-defined pathology and both the neurobehavioural deficits (Hameed et al., 2014). The main disadvantages in this model are concerns associated with the need for both craniectomy(Floyd et al., 2002), and anaesthesia (Silva et al., 2011, Bowles and Gold, 2012). According to Thompson, the lateral FPI model has emerged as the model most applicable to concussion and mTBI (Thompson et al., 2005, Lifshitz et al., 2007), additionally, fluid percussion can induce mild brain injury in mice of various ages (Shah et al., 2006, Titus et al., 2013, Zhang et al., 2013).

1.5. THESIS AIMS

There are many risk factors for mTBI, including the age at injury, sex, number of injuries and interval between injuries, however, details on how these factors affect the outcomes after mTBI are still inconclusive with many issues still to be resolved.

Whether the young are resilient or vulnerable to mTBI, if females are more or less vulnerable than males to mTBI, and whether a short time interval between injuries and additional injuries exacerbate mTBI pathology, are not fully understood. Animal models allow precise control of confounding factors including genetics, injury type and comorbidities. Therefore, the primary objective of this thesis is to further investigate the influence of the age at injury, sex and number of injuries and interval between them, on the cellular and subcellular responses after mTBI in a mouse model. A complimentary study seeks to the use a potentially multi target drug intervention to improve outcomes after mTBI. The current thesis is based on the hypotheses that the cellular and subcellular responses (including neuronal and glial changes) vary greatly depending on other risk factors, supporting the rationale for a more personalized medicine approach for the treatment of mTBI.

Aim 1: To discover the similarities and differences in cellular responses following mTBI in the young, adult and aged brains

Investigating the interplay between age at injury and mTBI is vital for understanding the vulnerability of different ages to mTBI and for suggesting the potential age dependent therapy.

To date, cellular changes following mTBI in a single species across the lifespan have been less well studied. Using immunohistochemical techniques Chapter 3 of this thesis aimed to investigate the similar and differential cellular response following mTBI in a Thy1-YFPH mice model.

Aim 2: To determine the therapeutic potential of the microtubule stabilizing agent epothilone D following mTBI in young and adult mice

Microtubule stabilizing agents have been proposed as potential treatment for TBI, spinal cord injury and neurodegenerative disease, in which epothilone D may be able to protect axons and dendrites, and inhibit the glial response. Dysregulation of neuronal microtubules after mTBI supports the potential treatment with microtubule stabilizing drugs. In Chapter 4 of this thesis, a low dose of microtubule stabilization drug epothilone D was administered in young and adult mice following mTBI in order to understand the therapeutic potential of epothilone D in different ages after brain injury.

Aim 3: To investigate the cellular changes following mTBI in male and female mice

It is still controversial as to whether males or females have better outcomes following mTBI in both animal and patient studies. Furthermore, females TBIs are less-studied compared to males. Chapter 5 of this thesis aimed to identify the similar and different cellular and subcellular responses after mTBI in both male and female Thy1-YFPH mice.

Aim 4: To investigate the cellular changes following an additional mTBI in a mouse model

There are increasing numbers of repetitive brain injuries across the world especially among children and adults involved in contact sports. However, cellular changes in animal studies modeling rmTBI are still inconclusive - while some found the number of injuries exacerbated the pathology, others did not. Chapter 5 of this thesis aimed to investigate the cellular changes following an additional mTBI administered 48 hours following the first mTBI in Thy1-YFPH mice.

2. MATERIALS AND METHODS

2.1. BREEDING AND GENOTYPING OF THY1-YFPH TRANSGENIC MICE

All experiments were approved by the Animal Ethics Committee of the University of Tasmania (ethics approval number A0015573) and were performed in accordance with NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2013. Studies were performed on young (1.5 months), adult (3 months) or aged (12 months) Thy1-YFPH transgenic mice. B6.Cg-Tg(Thy1-YFP-H)16Jrs/J (Stock #003782) mice were purchased from The Jackson Laboratory and maintained on a C57BL6 background. In these animals yellow fluorescent protein (YFP) is expressed under the control of the neuron-specific Thy1 promoter in approximately 80% of layer 5 and 2/3 neocortical pyramidal neurons (Fig 2.1) (Feng et al., 2000, Porrero et al., 2010). Ear punches were taken at weaning (4 weeks) to determine inheritance of the YFP transgene. Mice were housed in standard conditions (20℃, 12h/12h light/dark cycle), with access to food and water *at libitum*.

2.2. SURGERY PREPARATION

All surgical procedures were performed in a bio BUBBLE (FortCollinsco. CO, USA) with 80–100 HEPA filtered air circulations per hour. Mice were transferred to the surgical room, acclimatized for 30 min and weighed. The surgical bench and microscope was wiped down with isopropanol alcohol (IsowipeR, Kimberly-Clark, Australia). All surgical instruments were autoclaved. Mice were randomized to the injury group and sham group. All mice underwent identical surgery procedures. Briefly, mice were anesthetized in a plexiglass chamber with 5% isofluorane (Isoflo, Abbot Australasia Pty Ltd, Botany, NSW, Australia) in 100% oxygen. The head was secured in a stereotaxic frame (Narishige, Tokyo, Japan), with the body positioned on a warming pad (Stoelting, Wood Dale, IL, USA) to maintain body temperature during surgery. Once secured in the stereotaxic frame, surgical plane anesthesia (1.5-2% in oxygen) was maintained via nose cone and viscotears (Kingston-upon-Thames, Surrey, UK) was administered to the eyes. The scalp was cleaned with betadine (Sanofi-aventis Consumer Healthcare, Virginia, QLD, Australia) and 70% ethanol, and the topical anaesthetic bupivicaine (Bupivicaine hydrochloride, 50ul 0.25% in sterile saline; Pfizer, West Ryde, NSW, Australia) was administered under the scalp. A midline incision was made to expose the skull and a 3.0 mm craniotomy was made on the right parietal bone halfway between the sutural landmarks lambda and bregma. An injury hub was created by cutting off the metal end of a 22G needle (Livingstone international Pty Ltd, Rosebery, NSW, Australia) with a razor blade. The hub was attached to the skull with adhesive Loctite 454 (Henkel Australia, Sydney, NSW, Australia)

and dental cement (Heraeus Dental Science, Villebon, France) and was then filled with sterile saline. Pre-, peri- and post-surgical monitoring were performed to determine respiratory rate, confirm absence of reflexes and monitor mucous membranes/capillary refill time. Following application of the injury hub, mice were left to recover for one hour prior to application of the injury pulse.

2.3. LATERAL FLUID PERCUSSION BRAIN INJURY (LFPI)

In preparation for LFPI, each mouse was re-anesthetised in a pre-charged induction chamber containing 5% isoflurane in 100% oxygen. Following induction of anaesthesia, the animal was removed from the induction chamber, the hub was re-filled with sterile saline and attached to the FP302 Fluid Percussion Device (AmScien Instruments, Richmond, VA, USA; see Fig 2.2) via a 10cm spacing tube filled with sterile Milli-Q water. Briefly, a pendulum is released from a predetermined height, striking a piston connected to a chamber filled with saline solution, which generates a fluid pressure pulse against the intact dura. The mouse was placed on a heated pad and once a normal pattern of breathing resumed, but prior sensitivity to stimulation, an injury of mild severity was delivered to the intact dura by releasing the device's pendulum onto a fluid filled piston, causing transient displacement and deformation of the dura and underlying brain. A transducer incorporated into the device measured the pulse pressure and the peak pressure was recorded within the software. Following injury, mice were placed on their back and visually monitored for recovery of spontaneous breathing. Additionally, the time taken for animals to recover the righting reflex was recorded as a measure of transient unconsciousness/loss of consciousness (LOC). sham-operated mice underwent identical surgical procedures to brain injured mice and were connected to the injury device, however the pendulum was not released. Following injury/sham-operation, mice were re-anesthetized, the injury hub was removed and the incision sutured. Mice were observed for 2 hours (hrs) prior to return to their home cages and provided free access to food and water. For the first week following LFPI or sham-operation, animals were scored daily for signs of illness and stress and once a week thereafter.

2.4. IMMUNOHISTOCHEMISTRY AND STAINING OF MOUSE BRAIN TISSUE SECTIONS

All animals were deeply anesthetised with an overdose of sodium pentobarbital (300mg/kg; Troy Laboratories Pty Ltd, Smithfield, NSW, AU) and perfused transcardially with a fixative containing 4% paraformaldehyde (PFA) in 0.01M phosphate-buffered saline (PBS). The brains were removed and post-fixed in PFA overnight and then stored at 4℃ in 0.01M PBS containing 0.02% w/v sodium azide (Sigma Aldrich,Castle Hill, NSW, AU). Following overnight fixation,

the brains were embedded in 5% agarose and coronal sections with a thickness of 50 μm were generated using Leica VT1000S vibratome (Biosystems Australia Pty Ltd, Mount Waverly, VIC, AU). For immunohistochemistry, sections representing the impact site were placed serially into individual wells of a 24-well culture plate and processed as free floating sections. Sections were washed three times in 0.01M PBS then incubated in a solution of primary antibody diluted in the permeabilizing agent (0.03% Triton X-100) at room temperature for a period of 1 hr, and then transferred to 4℃ room for a period of 12 hrs. Primary antibodies included a rabbit anti-GFAP antibody (Z0334, 1:2000; Dako) or a rabbit anti-Iba-1 antibody (019-19741, 1:1000; Wako) to label astrocytes and microglia, respectively. After another three washes in PBS, the sections were incubated with Alexa Fluor® 568 donkey anti-rabbit conjugated secondary antibodies (A10042, 1:1000, Invitrogen) and 4', 6-diamidino-2-phenylindole (DAPI) (D3571, 1:6000, Invitrogen) in PBS at room temperature for 2 hrs. The reactions were completed by washing with PBS three times. Following completion of the immunohistochemistry protocol, sections were serially mounted onto slides for analysis.

2.5. MICROSCOPY AND IMAGE ANALYSIS

Images were collected with an UltraVIEW spinning disk confocal microscope running Volocity Software (PerkinElmer Australia Pty Ltd, Glen Waverley, VIC, AU), equipped with a 20x/0.5 air and Plan Apo 60x/1.20 water objective (Nikon, New York, NY). In order to get the same brain section of each mouse across groups, another investigator was appointed to choose the section where the two blades of the hippocampal dentate gyrus were clearly visible with DAPI staining. When performing the data analysis, I was blinded to all the animals. For quantitation of cortical thickness and YFP positive cell number and size, the microscope was configured to capture large stitched images of upper hemispheric quadrant of each brain with 20x objective (20um z-stacks, 1um slices). Quantification was performed in the entire upper ipsilateral quadrant of the neocortex (area outlined in red, Fig 2.1). For quantification of cortical thickness, three straight lines were manually drawn across images of the neocortex; one on the top end, one at the middle, and one on the bottom end. These measurements were averaged to get the overall cortical thickness. For quantification of YFP positive cell number and size, soma falling within this region were manually traced to determine average somal size as well as the number of soma per area of neocortex, or cell density of YFP positive neurons.

For quantification of astrocytic and microglial activation (the percentage of area occupied by GFAP expressing astrocytes and Iba-1 expressing microglia, respectively), the microscope was configured to capture large stitched images of the neocortical region of the injury site of

each brain with 20x objective (20um z-stacks, 1um slices). The neocortical impact site was manually traced in ImageJ (area outlined in yellow, Fig 2.1), and was converted to an 8-bit image. A threshold was applied to capture all staining and the percentage area of GFAP or Iba1 positive cells in the injured cortex was determined. Furthermore, in the current study, similar to other investigations, a counterstain hasn't been applied either for the imaging or analysis (Wang et al., 2018, Broussard et al., 2018). However, applying a nuclear counterstain for the analysis, such as DAPI, would have allowed a further assessment of number of microglia relative to all cells (Liu et al., 2018), in addition to the measures of 'number of cells' and 'percentage area of immunoreactivity' as reported.

Axon vulnerability was investigated within the Internal Capsule (IC), which is a large axonal tract directly under the pressure wave that is elicited in the LFPI model (outlined in blue, Fig 2.1). Axonal degeneration was quantified as the amount of axonal degeneration, the relative number of axonal fragments and the size of the axonal fragments in YFP positive axons. First, large stitched images of the internal capsule were captured with 20x objectives (20um z-stacks, 1um slices). The internal capsule was traced in ImageJ according to the mouse brain map (area outlined in blue, Fig 2.1), and was converted to 8 bit, and then was threshold to end of pixels. After seting the partical analysis (size from 10-10000, circularity from 0.3-10), the data of the percentage of area, the number of the particals and the average size of the particles were measured.

For dendritic spine density analysis, image stacks were captured with the 60x water objective (20um z-stacks, 0.2um slices) from layer 5 of the injured cortex. In Neurolucida (MBF Bioscienes, Willoston, VT, United States), dendrite and spine analysis was performed in Neurolucida. Dendritic shafts were traced from image stacks and spines were manually added onto the traced dendrites and categorized into mushroom (prominent head, thin neck), stubby (greater width than length), and thin (greater length than width) spines. Changes in dendritic spine density were determined by loading Neurolucida data files into Neurolucida Explorer™ (MBF Biosciences).

2.6. STATISTICAL ANALYSIS

All data was analyzed and graphs were created in GraphPad Prism (version 8.0, La Jolla, CA, United States), and all the data sets were first analyzed by normality tests (results in Appendix, page 124). If a data set fails the normality test, the logarithm or square root transformation were used to make the data normal distribution (de Carvalho et al., 2007). Then t tests or one-, two-, or three way analysis of variance (ANOVA) was used followed by Tukey's multiple

comparison test. Results were expressed as the mean ± standard error of the mean. In all cases, a *p*-value of <0.05 was considered significant. Statistically significant differences are noted on graphs where present. As it is not possible to perform a power calculation prior to commencing a largely exploratory hypothesis, n=6 animals was selected due to previous publications which found statistically significant results with similar numbers in similar experimental conditions (Zhu et al., 2003, Cutler et al., 2007, Suenaga et al., 2015, Ritzel et al., 2019). All figures were prepared in Adobe Photoshop CC (version 17.0.0, Adobe Systems, San Jose, CA, United States).

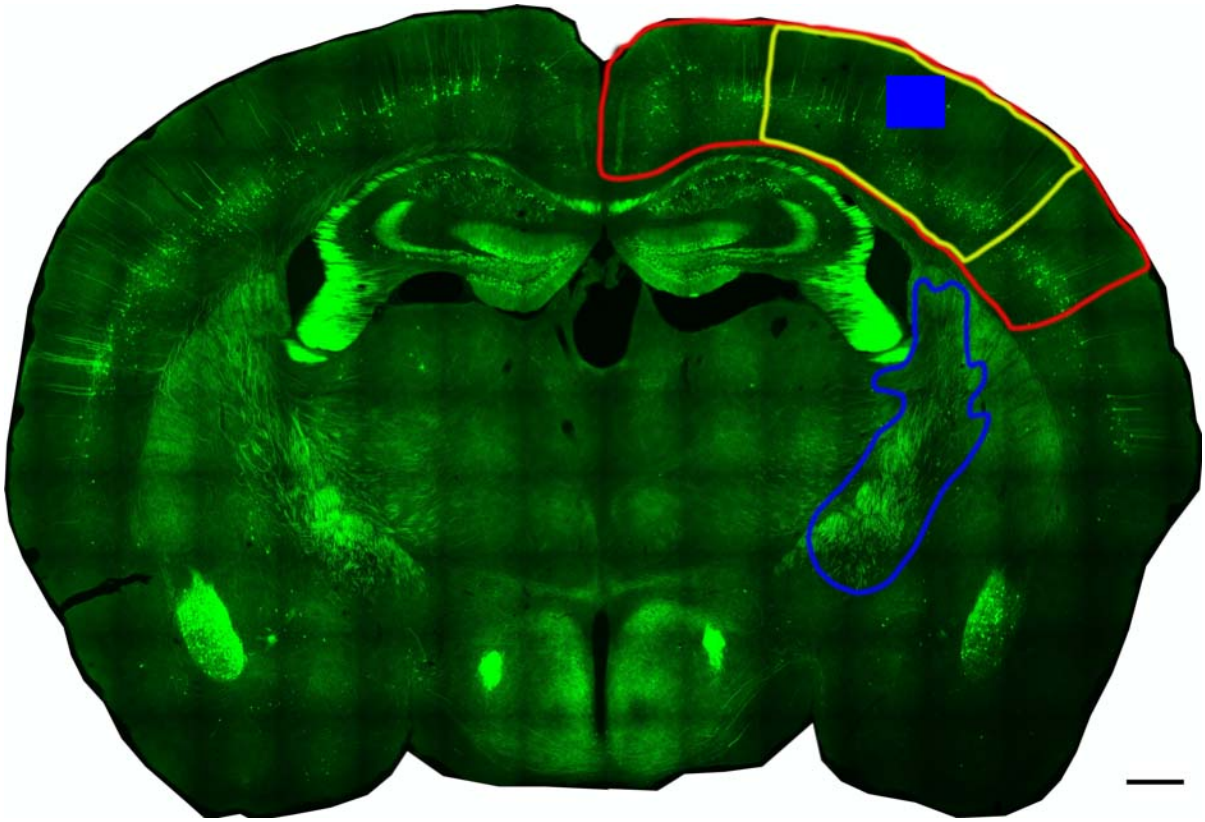

Figure 2.1. The coronal section of the Thy1-YFPH transgenic mouse brain.

Representative image of a coronal section of the Thy1-YPFH transgenic mouse brain. The area encompassed by the red line represents the area used for cortical thickness, somal size and cell density analysis. The area covered by yellow line represents the area used for the percentage area of glia analysis. Additionally, the area covered by blue box represents the area used for microglial morphology analysis. And the area covered by blue line represents the area used for axonal degeneration analysis. Scale bar= 400um.

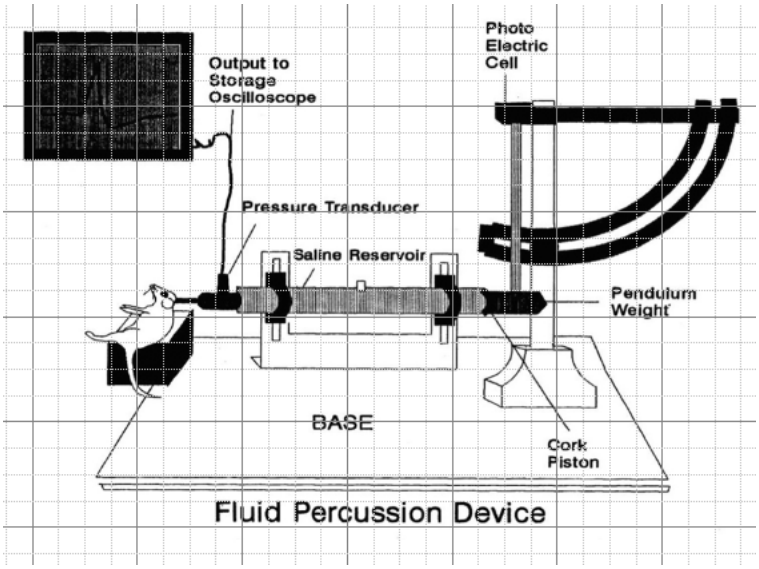

Figure 2.2 Lateral fluid percussion injury (Adapted from Lippert-Grüner et al., 2007)

3. THE CELLULAR RESPONSES TO MILD TRAUMATIC BRAIN INJURY ACROSS THE LIFESPAN

3.1. INTRODUCTION

mTBI, accounting for more than 80% of TBI cases (Luo et al., 2017, Dewan et al., 2018), is a serious public health issue with roughly 42 million people affected worldwide each year (Gardner and Yaffe, 2015). Clinically, mTBI is often caused by an impact or blast exposure to the head and may result in a brief alteration in consciousness or post-traumatic amnesia lasting less than 24h, loss of consciousness lasting less than 30 min, and a Glasgow Coma Score between 13 and 15 (Yu et al., 2017). With a growing ageing population, the number of aged mTBI cases is increasing. Previous studies have suggested that increased age at the time of injury is associated with higher mortality and morbidity, as well as worse behavioural and cognitive impairments. However, the underlying pathophysiological mechanisms which contribute to worse outcomes after TBI with ageing are less well studied and remain poorly understood.

The cellular responses are vital to the pathological and functional changes after TBI. Animal studies have shown mTBI is not associated with overt cortical loss. Overwhelming evidence however indicates that one of the major pathological features of mTBI is widespread axonal disruption, particularly in the white matter tracts (Sword et al., 2013, Hånell et al., 2015, Katz et al., 2015, Mierzwa et al., 2015, Sinha et al., 2017, Vascak et al., 2018). Experimental studies have also shown that subtle axonal damage manifests acutely following injury, with the potential to progress over subsequent years (Johnson et al., 2013a, Johnson et al., 2013b). Furthermore, results from both clinical and experimental studies have found glial activation during acute, subacute and chronic phases following brain injury, which may have both beneficial and detrimental implications for the axonal damage and recovery (Kou and VandeVord, 2014). Despite achieving a greater understanding of the vulnerability of the brain to diffuse axonal damage and glial activation in mTBI, it remains unclear how age at the time of injury affects axonal pathology and glial reactions following mTBI and the sequence of events that lead to the lasting axonal and glial changes.

The ageing brain is characterized by a number of different properties, including brain atrophy, decreased brain plasticity, higher oxidative stress, metabolism disruption, and a pro-inflammatory state (Craft, 2005, Yankner et al., 2008, Romano et al., 2010, Pannese, 2011, Popa-Wagner et al., 2011, Petralia et al., 2014, Seib and Martin-Villalba, 2015). Clinical and experimental studies suggest that outcomes following TBI worsen with age (Durham et al.,

2000, Thompson et al., 2006, Onyszchuk et al., 2008, Rowe et al., 2016, Yamagami et al., 2019). With regard to young animals, the brain is still undergoing development and typically displays relatively high levels plasticity. Furthermore, abundant neural stem cells, quicker amyloid beta debris clearance, and quicker metabolism recovery in young animal brains have been observed (Venkitaramani et al., 2007, Deng-Bryant et al., 2011, Zhang et al., 2017b). However, clinical studies have demonstrated that the immature brain may actually be more vulnerable, rather than resilient, to brain injury (Kirkwood et al., 2006, Donders and Warschausky, 2007, Lichte et al., 2015, Keenan et al., 2018, Karr et al., 2019). Thus, it is still inconclusive how age at the time of injury influences recovery from mTBI. Although many animal studies have reported the sequela of mTBI at distinct time-points in the lifespan (Sword et al., 2013, Kou and VandeVord, 2014, Hånell et al., 2015, Mierzwa et al., 2015, Vascak et al., 2018), few have investigated the cellular response to a mTBI received at critical points throughout the lifespan in a single species, using a consistent animal model. To address this gap, we used the mild lateral fluid percussion brain injury model to determine the evolution of cellular pathology at critical points across the lifespan in a mouse model.

3.2. MATERIALS AND METHODS

Studies were performed on young (1.5 months), adult (3 months) and aged (12 months) Thy1-YFPH transgenic male mice. Mice were randomized to the injury group (n=6 young FPI 1wk, n=6 young FPI 4wk, n=6 adult FPI 1wk, n=6 adult FPI 4wk, n=5 aged FPI 1wk, and n=6 aged FPI 4wk), sham group (n=5 young sham 1wk, n=6 young sham 1wk, n=6 adult sham 1wk, n=6 sham 4wk, n=6 aged sham 1wk, and n=6 aged sham 4wk) and naïve group (non-injured, n=6 young, n=5 adult, and n=6 aged) groups. Lateral fluid percussion injury (LFPI) of mild severity (1.40±0.01 atmospheres) was delivered to the intact dura by releasing device's pendulum onto a fluid filled piston, causing transient displacement and deformation of the dura and underlying brain. Finally, mice were perfused at either 1 week or 4 weeks post injury. Naïve mice were perfused at 1.5 and 3 months and 12 months of age to match their young, adult and aged brain-injured and sham-operated counterparts. Details regarding immunohistochemistry protocols, imaging and analysis can be found in Chapter 2. Iba-1 was chosen as it is a traditional, well-defined marker and has been widely used to label microglia/macrophages (Imai et al., 1996). Additionally, Iba-1 labelling has been used to perform further analysis of changes in microglial morphologies in response to trauma and disease, specifically to investigate microglial activation (Hovens et al., 2014, Heindl et al., 2018).

For quantification of microglial morphology, image stacks were captured with a 40x objective (1um slices; 20 slices, flattened to a single z-stack image for analysis) at the impact site (Fig

2.1). One 40x image was taken in a section in each animal, in total there were n=72 images for analysis. Using an analysis protocol based on the work of Rusconi and colleagues, a threshold was applied to captured images (n= 72) such that the individual branches of the microglia could be clearly identified. Microglial activation was determined by scoring every microglia in the 40x z-stack as either ‘resting’ (cells with small, round cell bodies and elongated, thin processes) or ‘activated’ (cells with bigger, less circular cell bodies and dense ramifications of variable thickness; or cells with big, irregular cell bodies and only a few, shorter, and poorly ramified processes; or cells with an ovoid‐shaped body devoid of extensions or with one or two thick, unramified processes) (Rusconi et al., 2018). The characterization into these categories was consistently applied, and all images were blinded for analysis. In order to use consistently the same brain sections for analysis across groups, another investigator was appointed to choose the section where the two blades of the hippocampal dentate gyrus were clearly visible with DAPI staining. The specific site or region of interest directly at the center of the injury site, as could be seen in Fig 2.1 labelled with blue box (the injury area could be clearly identified under microscope, as the trace of craniotomy window above the neocortex surface could be observed), was consistently selected for image analysis (n=1 per section/animal, 6 animals per groups, 72 images in total analyzed). The investigator was blinded to the injury status of all animals. Microglia were classified as either ‘resting’ or ‘activated’ in order to further understand the glial response to trauma. Statistically significant differences are noted on graphs where present.

3.3. RESULTS

3.3.1. Ageing is associated with thinning of the neocortex, increased glial activation and axonal degeneration

In the present study, cortical thickness was reduced in non-injured aged brains relative to non-injured young and adult brains (Fig 2.1 A). In the neocortex, YFP positive cell density and soma size were increased in aged brains compared to young and adult brains (Fig 2.1 B, C; p=0.0011 and p=0.0060, respectively). Astrocyte activation was higher in aged mice relative to young and adult mice (Fig 2.1 F; p=0.0087). Additionally, microglial activation was increased in aged mice compared to young and adult mice (Fig 2.1 G; p=0.0011 and *p=0.0003, respectively*). In the internal capsule white matter tracts, axonal degeneration was higher in the aged compared to young and adult brains (Fig 2.1 H; p=0.0004, p<0.0001, respectively). Furthermore, the relative number of axonal fragments was found to be higher in the aged compared to young and adult brains (Fig 2.1 I; p=0.0261, p=0.0044, respectively). The size of the axonal fragments was higher in the aged compared to young and adult brains (Fig 2.1 J; p=0.0325, p=0.0061, respectively). Total spines density and density of individual spine sub-classes (thin, stubby and mushroom) did not differ in the cortex of aged mice compared to either young or adult mice (Fig 2.1 K-N; p=0.2273, p=0.0512, p=0.2348, p=0.7103, respectively). Overall, the aged animals were characterized as having a thinner neocortex, as well as an increased glial response and higher level of axonal degeneration compared to younger mice.

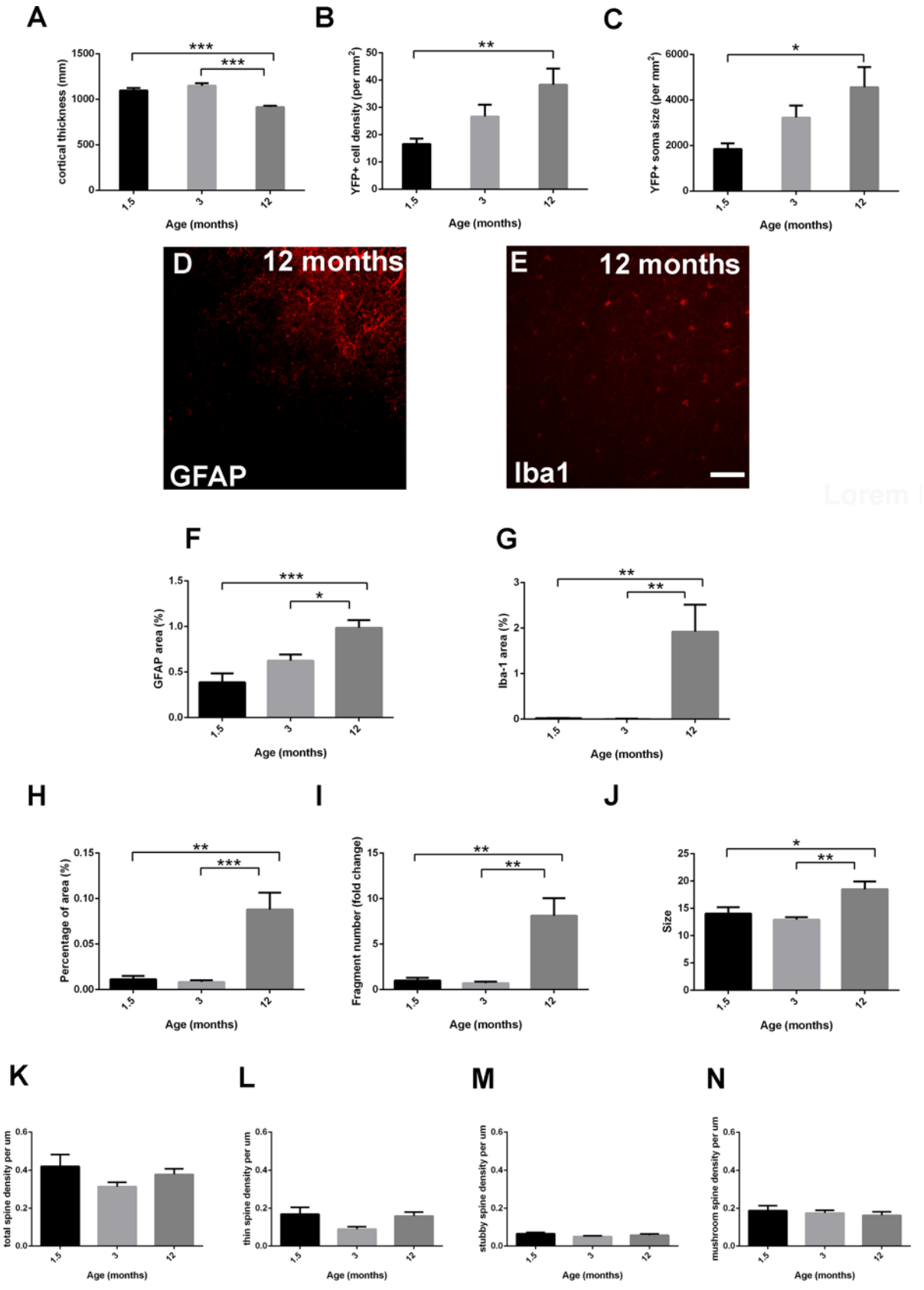

Figure 3.1. Ageing is associated with increased glial activation and axonal degeneration.

*The cortical thickness of aged (12 months) animals was reduced compared to both young (1.5 months) and adult (3 months) animals (A). YPF positive cell density (B) and cell soma size (C) was higher in the aged animals compared to young animals. Representative image of aged (12 months) GFAP (D) and Iba1 (E). Both the astrocytic activation (F) and microglial activation (G) were higher in the aged compared to young and adult animals. The percentage area of axonal degeneration (H), the fragmentation number (I), and the average size of axonal fragmentation (J) were higher in the aged animals compared to both the young and adult cohorts. The total spine density and the spine sub-class density did not differ between different age groups (K-N). Data are presented as mean ± SEM. One-way ANOVA, followed by Turkey's tests *p<0.05, **p<0.01, ***p<0.001, ****p<0.001. Scale bar=50um.*

3.3.2. LFPI model generated a reproducible mTBI in the young, adult and aged mouse cohorts

mTBI in the mouse is considered as a mortality rate of 0-5%, a righting reflex of 2-4min, and a loss of consciousness of ~2min (Morehead et al., 1994, Alder et al., 2011). In the current study, no mortalities were observed, however, LFPI was immediately followed by a short period of apnoea independent of age at injury (Fig 3.2 B; p=0.5720), and a delay of righting reflex time compared to sham-operated controls (Fig 3.2 C; $F_{1,63}$ =151.5; p<0.0001) independent of age at injury (Fig 3.2 C; $F_{2,63}$ =1.200; p=0.3079). Gross morphological analysis indicated there was no overt cortical loss or cavitation after injury, furthermore, quantitative analysis showed the injury did not change the cortical thickness (Fig 3.2 D; $F_{1,61}$ =0.0958, p=0.7580), the YFP positive cell density (Fig 3.2 E; $F_{1,61}$ =1.352; p=0.2494) or the soma size (Fig 3.2 F; $F_{1,62}$ =1.116, p=0.2948) compared to the sham-operated controls at either 1 week or 4 weeks post injury in the 1.5, 3 and 12 month old mice. Overall, the LFPI represented mild injury across the lifespan in this study.

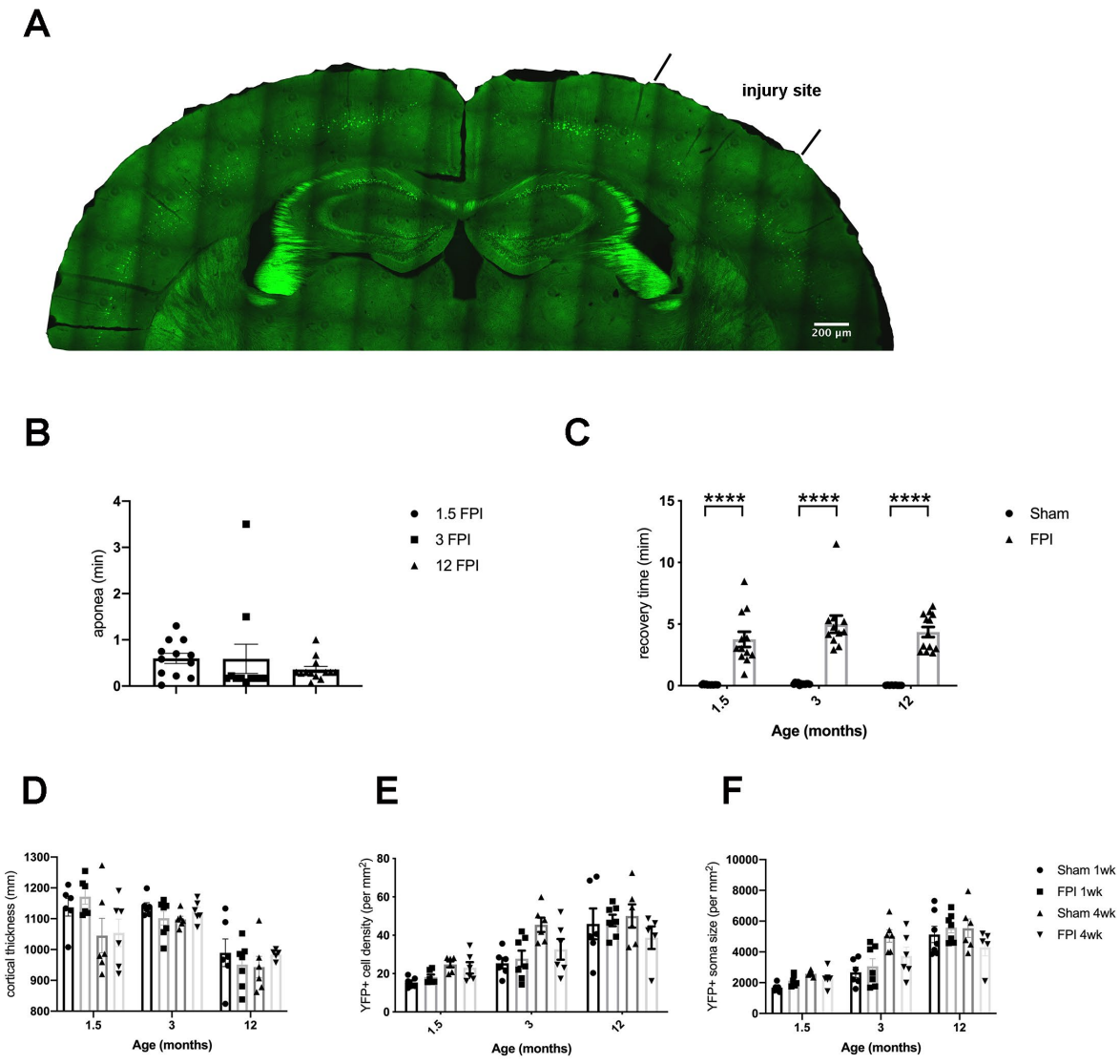

Figure 3.2 LFPI model generated a reproducible mTBI in young, adult and aged mice.

*Representative image of LFPI in an adult Thy1-YFPH mouse brain (A). LFPI caused a short period of apnoea in young, adult and aged mice, with no difference between ages (B), one-way ANOVA. The injury caused significantly increased righting reflex time in young, adult and aged mice, and there was no difference between ages (C), two-way ANOVA. The injury did not cause obvious change in the cortical thickness (D), YFP positive cell density (E) or cell soma size (F) across the lifespan, three-way ANOVA. Data are presented as mean ± SEM. ****p<0.0001. Scale bar=200um.*

3.3.3. Astrocytic GFAP upregulation in the cortex after mTBI peaks in mice at 1.5 months of age

The consequence of mTBI on GFAP immunoreactivity was investigated within the neocortical region of the injury site (experimental details can be found in Chapter 2) (Fig 3.3 A). The percentage of area covered by GFAP immunoreactivity was investigated over the timecourse. An ordinary three-way ANOVA revealed a significant injury by week post injury interaction ($F_{1,59}$ =32.91, p<0.0001). Tukey's multiple comparisons tests identified that percentage of GFAP immunoreactivity was significantly increased at 1 week FPI in comparison to 1 week sham in animals of 1.5 months old at the time of injury (Fig 3.3 B; p=0.0039). However, this activation was not observed in either 3 or 12 month cohort. Furthermore, the GFAP immunoreactivity in injured brains was significantly resolved from 1 week post injury to 4 weeks post injury in animals 1.5 month (p=0.0497) and 12 months (p=0.0473) of age at injury. Overall, this data indicates that the astrocytic response to mild injury occurs early and is resolved. Furthermore, this data indicates that adult (3months) mice do not undergo this astrocytic response.

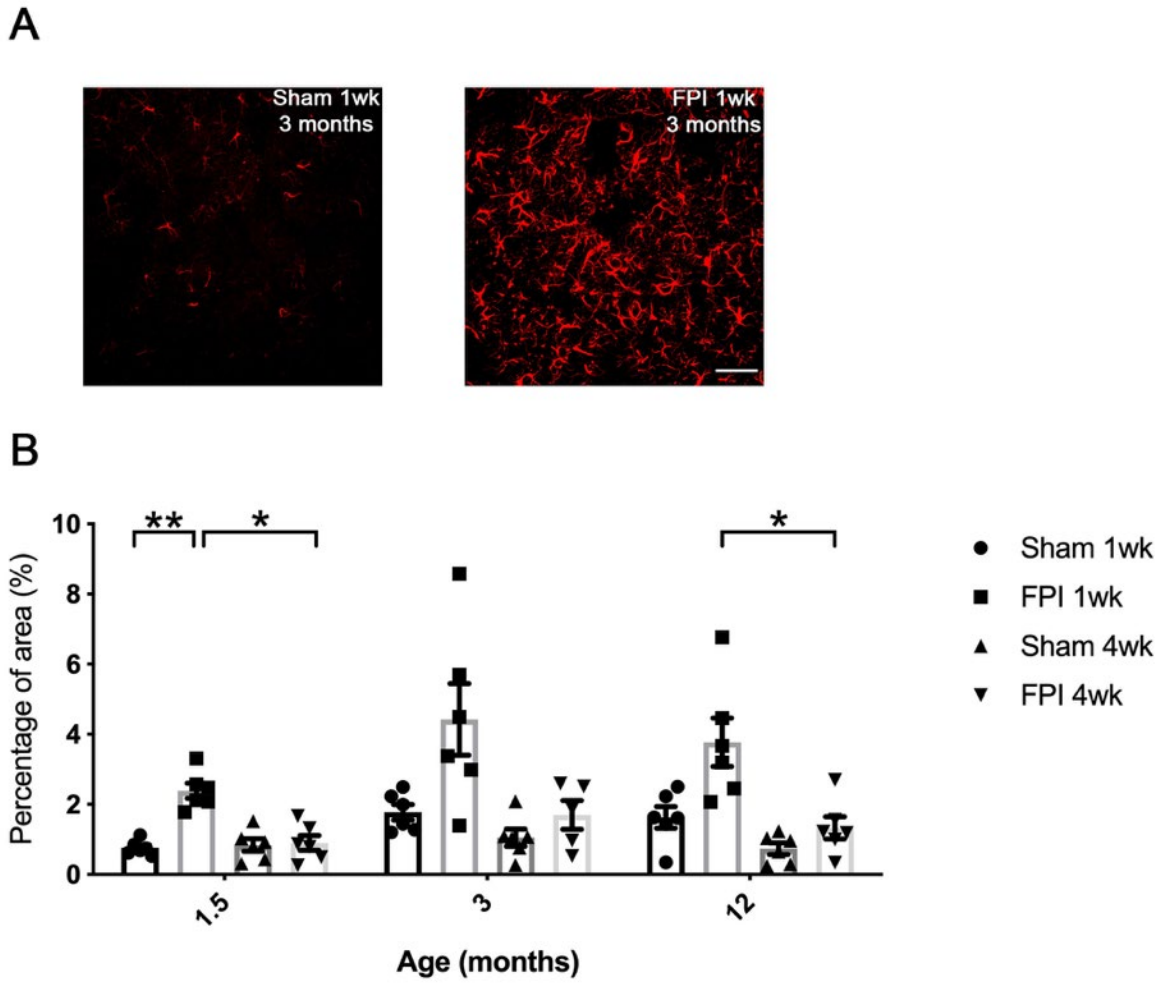

Figure 3.3. Astrocytic GFAP upregulation in the cortex peaks at 1 week following mTBI in 1.5 month old animals.

*Representative images of astrocyte activation in sham and FPI mice, within the neocortical region of the injury site (A). The percentage of GFAP immunoreactivity was significantly increased at 1 week FPI in comparison to 1 week sham in 1.5 months of age animals. Additionally, the percentage of GFAP immunoreactivity at 1 week FPI was significantly decreased compared to 4 week FPI in animals at both 1.5 months and 12 months of age (B). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests *p<0.05, **p<0.01. Scale bar=50um.*

3.3.4. Microglial Iba1 upregulation in the cortex is dependent on age at injury and time following mTBI

The consequence of mTBI on Iba1 immunoreactivity was investigated within the same region of analysis as GFAP (experimental details can be found in Chapter 2) (Fig 3.4 A). Iba-1 is expressed not only in activated microglia but also in resting microglia and in circulating macrophages in the brain (Walker and Lue, 2015). Therefore, Iba-1 expression is expected to be found in both sham operated mice and brain injured mice, albeit at lower levels, as evident in Fig 3.4 A. The percentage of area covered by Iba1 immunoreactivity was investigated over the timecourse. An ordinary three-way ANOVA revealed a significant injury by week post injury interaction ($F_{1,59}$ =60.95, p<0.0001). Tukey's multiple comparisons tests identified that percentage of Iba1 immunoreactivity was significantly increased at 1 week FPI in comparison to both 1 week and 4 week sham in animal of 3 months old at the time of injury (Fig 3.4 B; p=0.0011, p<0.0001 respectively). Iba1 immunoreactivity was significantly (*p<0.0001*) increased in the 12 month cohort at both time points post injury in comparison to animals injured at 1.5 months (1 week sham p<0.0001, 1 week FP1 *p<0.0001*, 4 week sham p=0.0001, 4 week FPI p<0.0001) (Fig 3.4 B). To determine if this alteration in microglia Iba1 immunoreactivity was specific to a certain subtype of microglia, the number of resting and activated microglia were scored (Fig 3.4 C). For the resting phenotype, an ordinary three-way ANOVA determined that there was a significant age and injury interaction (Fig 3.4 D; $F_{2,51}$ =5.057, p=0.0059). Tukey's multiple comparison test determined that there was a significant decrease in resting microglia in 12 months of age, 1-week post-injury, in comparison to 3-month old's (p=0.0012) and in 12 months old's, 4 weeks post-injury in comparison to 1.5-month old's (p=0.0201). For the activated phenotype, an ordinary three-way ANOVA determined that there was a significant age and injury and time interaction (Fig 3.4 E; $F_{2,67}$ =45.57, p<0.0001). Tukey's multiple comparison test determined that there was a significant increase in activated microglia at 12 months of age, 1 week post-injury, in comparison to 1.5 (p<0.0001) and 3 months (p=0.0003) of age and at 4 weeks post-injury in comparison to 1.5 months (p<0.0001) and 3 months (p<0.0001) of age. Furthermore, TBI significantly increased the number of activated microglia at 12 months of age in comparison to 1 (p<0.0001) and 4 weeks (p<0.0001) sham. Overall, this data indicates that the Iba1 response to injury is dependent upon age, and that the 12-month age has an altered microglia profile.

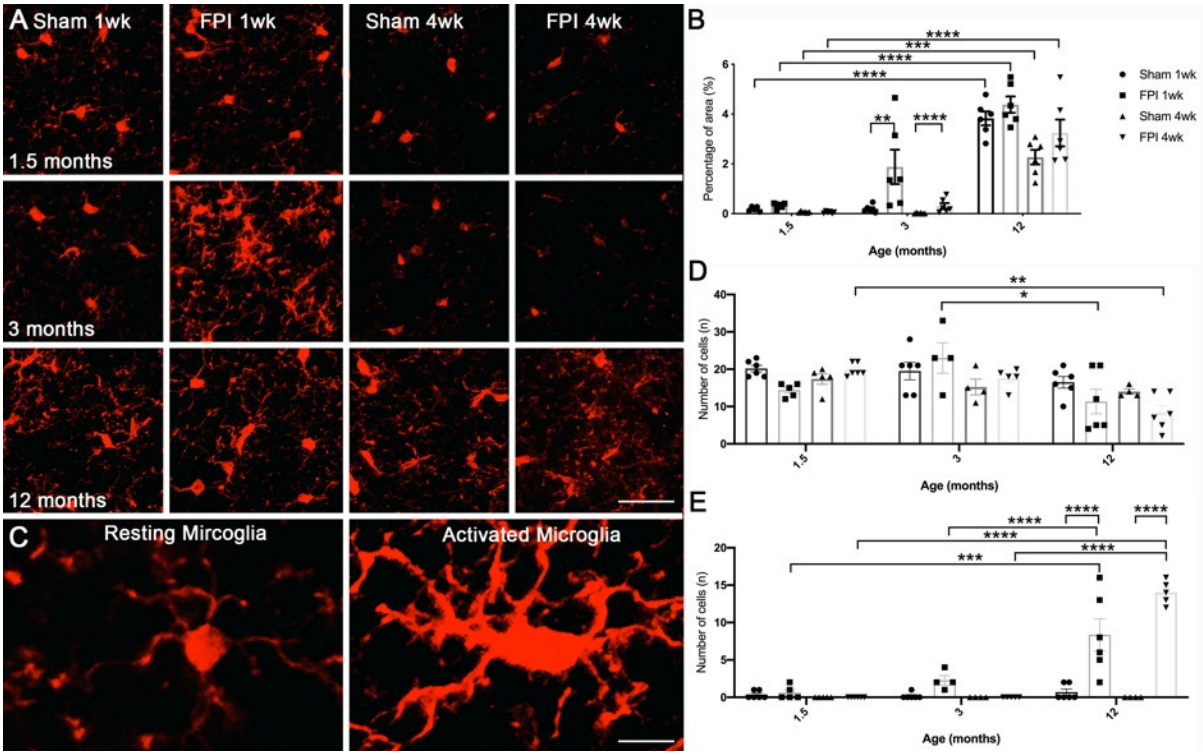

Figure 3.4. Microglia Iba1 upregulation in the cortex is dependent upon age at injury and time post injury.

*Representative images of microglial activation in sham and FPI mice, within the neocortical region of the injury site (A). The percentage of Iba1 immunoreactivity was significantly increased at both 1 week and 4 week FPI in comparison to the corresponding shams in animals 3 months of age at injury (B). The percentage of Iba1 immunoreactivity was significantly increased in all cohort of animals at 12 months of age compared to 1.5 months of age (B). Representative images of resting microglia and activated microglia (C). The number of resting microglia in brain injured mice at 1 week post injury was significantly decreased in 12-month mice in comparison to 3 months at injury, and at 4 weeks post injury was significantly decreased in 12 months compared to 1.5-month-old at injury (D). The number of activated microglia was significantly increased at both 1- and 4-weeks post-injury in 12-month-old mice, in comparison to the corresponding shams. Additionally, the number of activated microglia was significantly increased at 1- and 4-weeks post-injury in 12-month-old mice compared to mice at 1.5 and 3 months of age (E). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar=40um.*

3.3.5. Axonal degeneration in the internal capsule after mTBI is dependent on the age at injury and time post injury

The association between age at injury and axonal vulnerability was investigated within the Internal Capsule (IC) (Fig 3.5 A). This is a large axonal tract directly under the pressure wave that is elicited in the LFPI model. First the total amount of axon degeneration was quantified as a percentage of total area (0% degeneration would be all axons intact) (experimental details can be found in Chapter 2) (Fig 3.5 B). An ordinary three-way ANOVA revealed a significant interaction between week post injury and age at injury ($F_{2,50}$ =4.430, p=0.0173). Tukey's multiple comparisons tests identified multiple significant differences. The degree of axonal degeneration was significantly increased at both 1 week and 4 weeks post injury in comparison to shams in animals injured at 3 months of age (p=0.0012, p<0.0001 respectively) and at 12 months of age (p=0.0004, p<0.0001 respectively). .There was significantly more axonal degeneration at 1 week post injury compared to shams in animals injured at 1.5 months of age (p<0.0001). Furthermore, at 4 weeks post injury, in animals injured at 3 months (p<0.0001) or 12 months of age (p<0.0001) in comparison to those that were 1.5 months at the time of injury, indicating that axonal degeneration increased with increasing age.

Next, the relative number of axonal fragments was quantified (Fig 3.5 C). An ordinary three-way ANOVA revealed a significant interaction between week post injury and age at injury ($F_{2,49}$ =5.550, p=0.0067). Tukey's multiple comparisons tests identified multiple significant differences. The number of axon fragments was significantly higher at both 1 week and 4 weeks post injury in comparison to shams in animals injured at 3 months of age (p=0.0058, p=0.0005 respectively) and 12 months of age (p=0.0458, p=0.0028 respectively). Additionally, the number of axonal fragments was significantly increased at 1 week post injury compared to shams in animals injured at 1.5 months of age (p<0.0001).

Finally, the size of the axonal fragments was investigated (Fig 3.5 D). An ordinary three-way ANOVA revealed a significant interaction between week post injury and age at injury ($F_{2,50}$ =6.325, p=0.0036). Tukey's multiple comparisons tests identified multiple significant differences. The size of axonal fragments was significantly increased at 1 week post injury in comparison to shams in animals injured at 3 months of age (p=0.0188). Overall, this data indicates that the axonal response to injury changes over time in a differential manner depending upon age. Specifically, in mice aged 1.5 months at the time of injury axonal degeneration improves over time, however in mice aged 3 months and 12 months at injury, axonal degeneration does not resolve and instead worsens over time.

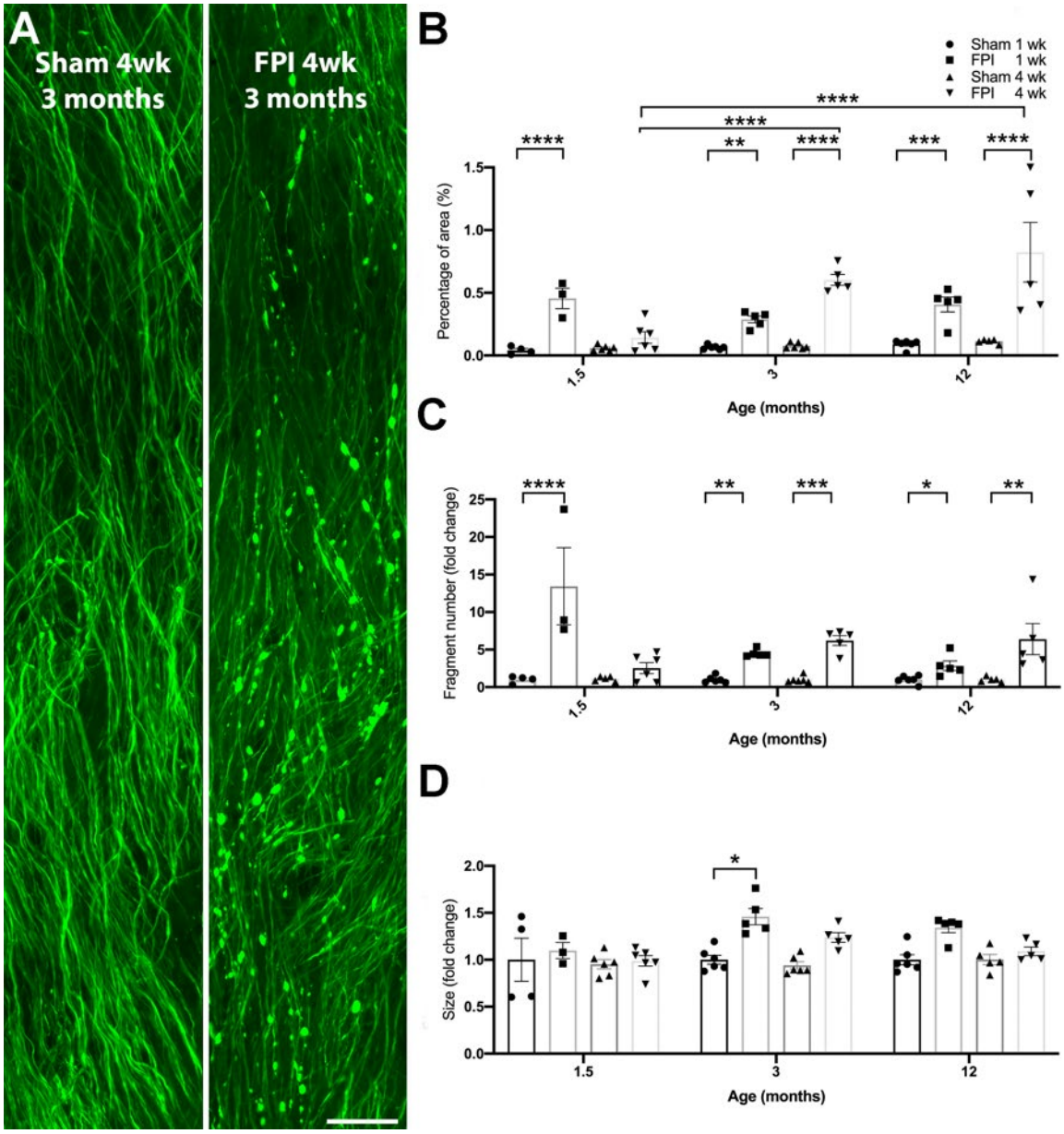

Figure 3.5. Axonal degeneration in the internal capsule is dependent upon injury, time and age following mTBI.

*Representative image of mTBI on axons in the internal capsule of the Thy1-YFPH trangenic mouse brain (A). The total amount of axon degeneration was significantly higher at 3 months and 12 months in comparison to 1.5 months (B). Moreover, the number of axonal fragments was significantly increased at both 1 week and 4 weeks in comparison to shams in mice injured at 3 months and 12 months (C). Finally, the size of the axonal fragments was higher at 3 months (D). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar=40um.3.3.6. Spine density did not change after injury independent of age at injury or time post-injury.*

3.3.6. Spine density did not change after injury independent of age at injury or time post-injury

In the present study, there was no significant difference between total spines density in injured animals compared to shams in the 1.5, 3 or 12 month old cohorts, at either 1 week or 4 weeks post injury (Fig 3.6 B; $F_{1,61}$ =1.579, p=0.2136). Similarly for each individual morphological dendritic spine subtype, there were no significant interactions between spine density, age at injury or time post for injury for thin (Fig 3.6 C; $F_{1,61}$ =0.5184, p=0.4743), stubby (Fig 3.6 D; $F_{1,61}$ =0.8654, p=0.3559), or mushroom spines (Fig 3.6 E; $F_{2,61}$ =1.960, p=0.1666). Overall, the mild injury did not change total spines density or the density of individual spine sub-classes across the lifespan.

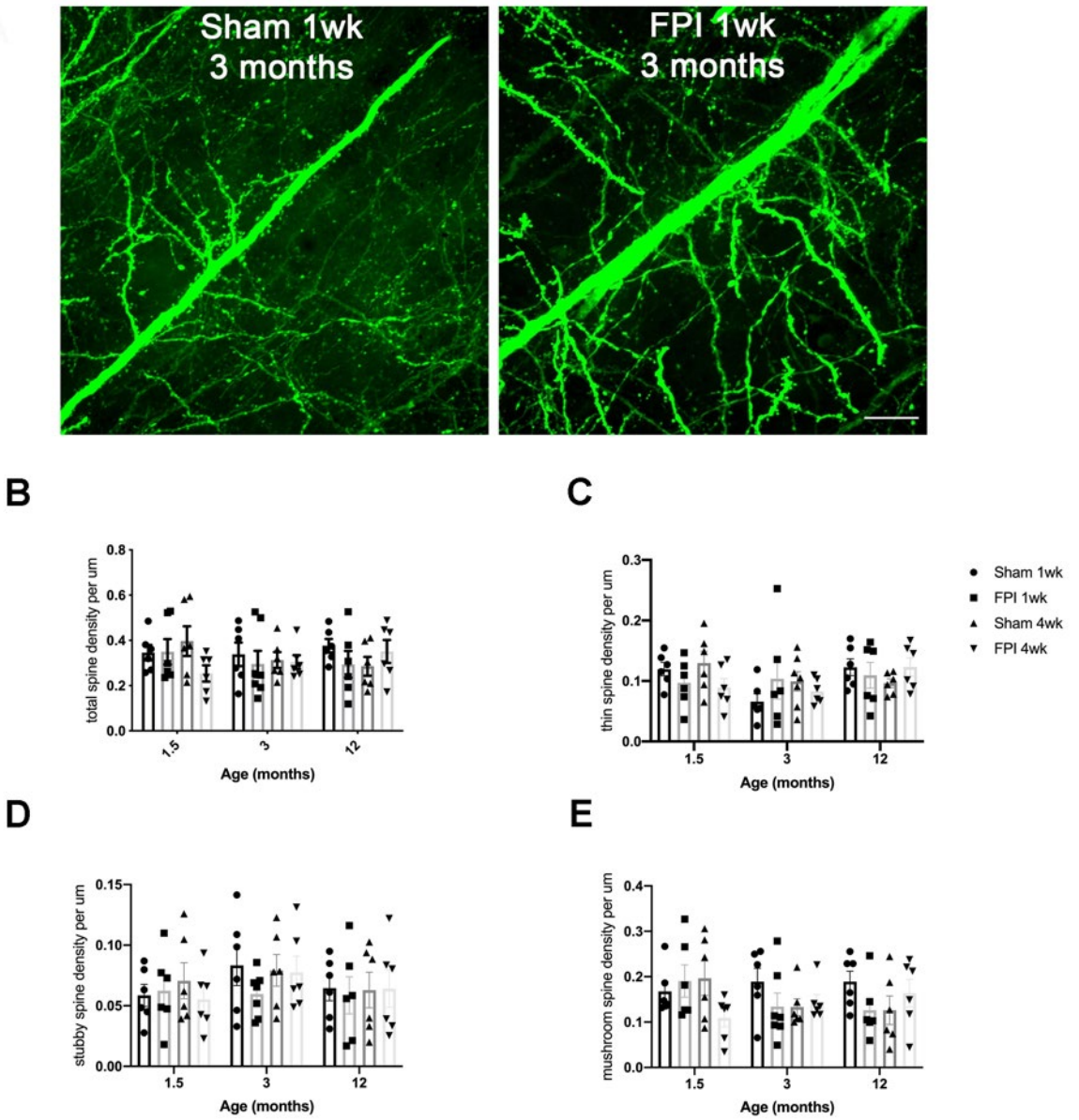

Figure 3.6. Spines density did not change after mTBI.

Representative images of mTBI on spine density in the injury site of the Thy1-YFPH mouse brain (A). No significant change was found between total spine density (B), as well as the density of thin (C), stubby (D) and mushroom (E) spines in injured animals compared to shams in the 1.5 months, 3 months or 12 months old cohorts, at either 1 week or 4 weeks post injury. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests. Scale bar=10um.

3.4. DISCUSSION

According to the Centers for Disease Control and Prevention (CDC), children aged 0-4 years, adolescents aged 15-19 years, together with older aged over 75 years have a high incidence rate of TBI (Laskowitz and Grant, 2016). Increased mortality is reported in the aged relative to adolescents and adults following moderate and severe TBI in both animal and clinical studies (Hamm et al., 1991, Timaru-Kast et al., 2012, Cheng et al., 2014b). Additionally, it has been postulated that brain atrophy in the aged brain may result in augmented diffuse axonal injuries and reduced neurorepair capacity (Peters, 2006, Thompson et al., 2006, Antona-Makoshi et al., 2015). With regard to the young population, they are generally recognized higher as having capacity for neuro-plasticity (Mundkur, 2005, Staudt, 2010, Kolb and Gibb, 2011, Kolb et al., 2013), however, it has also been reported that young individuals exhibit worse functional outcomes following moderate to severe TBI compared with older children and adults (Prins et al., 1996, Anderson et al., 2005, Emami et al., 2017), and persist with post-concussive symptoms from weeks to years after mTBI (Gagnon et al., 2009, Barlow et al., 2010, Taylor et al., 2010, Anderson et al., 2017). With regard to mTBI, brain injuries in the aged are less well studied, and it is still under debate whether younger age at the time of injury is associated with a better or worse outcome following mild injury. Thus, we investigated specific aspects of neuronal and axonal integrity as well as glial activation at discrete time-points throughout the lifespan to characterize the injured-brain during acute and chronic phases after injury. In the current study, we demonstrated aged (12 months) non-injured brains were characterized by a thinner cortex, increased glial activation, as well as higher axonal degeneration relative to young and adult brains. Following mTBI, we found age dependent microglial activation over time with aged injured animals having higher microglial activation. Furthermore, we also observed age at injury and time post injury dependent axonal degeneration, with adult (3 months) and aged (12 months) animals having higher axonal degeneration after injury compared to shams, with these axonal changes evolving over time.

To investigate brain changes that occur over the ageing process, we focused on cellular changes, specifically glial activation and axonal degeneration. In accordance with previous clinical and experimental findings (Sandhir et al., 2004, Popa-Wagner et al., 2011, Salvadores et al., 2017), we observed that the aged (12 months) brains had a thinner neocortex and increased pyramidal neuron density in the cortex than both young (1.5 months) and adult (3 months) brains, as well as higher astrocytic and microglial activation. The increased pyramidal neurons density and soma size may due to decreased cortical thickness, as it has been suggested there is no overt neuron loss in the ageing process (Egawa et al., 2016). Preceding animal studies have observed morphological changes with larger axons and less axons in the

optic nerve of aged mice (Adalbert and Coleman, 2013, Stahon et al., 2016). In our study, we found that the axonal degeneration in the internal capsule was higher in the aged brain compared to younger brains, which may underlie the functional deficits observed in the aged. In contrast to previous findings using Golgi staining and fast blue in the prefrontal cortex (Cupp and Uemura, 1980, Kabaso et al., 2009), we did not find any age-related spine density changes, which may be due to the different method for analysis used.

The lateral fluid percussion injury model has been widely used to mimic clinical mTBI pathology (Spain et al., 2010, Alder et al., 2011, Shultz et al., 2011). mTBI did not result in gross cortical loss or cavitation in clinical and pre-clinical studies (Iverson et al., 2010, Gao and Chen, 2011, Meconi et al., 2018). Moreover, mild injury is associated with low mortality rate (0-5%), a loss of consciousness (~2min), and a delay of righting reflex (2-4min) (Morehead et al., 1994, Alder et al., 2011, Teng and Molina, 2014, Katz et al., 2015). In the current study, results of mortality, loss of consciousness and delay righting reflex suggest that the injury used was mild in severity, this was also supported by no significant changes in either the neocortex thickness or neuronal population density of cell size across the lifespan. Furthermore, Hamm and colleagues have reported longer righting response time in aged (20 months old) relative to young (3 months old) brain injured mice (Hamm et al., 1991), however, we observed the period of unconsciousness and righting reflex time in aged brain injured mice did not differ from younger brain injured mice. This discrepancy may due to the differences in injury severity, as injury used in Hamm's study, as the injury pulse was between 1.7 to 1.8 atm and 20% mortality and 50% mortality was reported in the study.

Kou and VandeVord reported that glia are acutely activated after TBI and have been proposed to have both beneficial and detrimental effects (Kou and VandeVord, 2014). Unlike previous studies (Goodrich et al., 2016, Rodriguez-Grande et al., 2018), we did not observe that the mild injury significantly change activated astrocytes in the injured cortex in either adult or aged brains at either 1week or 4 weeks post-injury, this may due to insufficient numbers of adult and aged injured brains. Furthermore, in contrast to previous findings (Tzekov et al., 2016, Ferguson et al., 2017b, Mouzon et al., 2018b), we did not observe the highest astrocytic activation after injury in the aged cohort, which may be relevant to the injury severity and site measured. Logsdon and colleagues reported increased GFAP mRNA and protein expression has been observed at earlier stages after injury (2 days and 3 days post injury) (Logsdon et al., 2017). In the current study, astrocyte activation was observed after injury in the young brain injured mice compared to sham operated mice, at later stages, astrocytic activation was resolved at 4 weeks post-injury in young brain-injured mice. Microglial activation after mTBI has been observed in the corpus callosum and in the thalamic sites (Lafrenaye et al., 2015,

Goodrich et al., 2016). Consistent with previous animal studies (Lafrenaye et al., 2015, Goodrich et al., 2016), in the current study, we also observed mild injury significantly activated microglia within the injured cortex in adult brains at both 1 week and 4 weeks post-injury. However, we did not observe injury related microglial activation in either adult or aged animals. The underlying reason may be due to the resolution of injury related microglial activation in adult and aged animals was earlier compared to young animals, thus, investigation at earlier post injury time points is warranted in future studies. Consistent with previous animal studies (Kumar et al., 2013, Ferguson et al., 2017b, Cheng et al., 2018), we observed that microglial activation was higher in aged injured brains compared to young injured brains. In order to further understand the role of activated microglia, further studies classifying microglial phenotypes and analyzing cytokines as well as chemokine are warranted.

Previous studies have observed axonal morphological and functional changes in the brain after mTBI, most of them investigated the injured cortex and the corpus callosum (Mierzwa et al., 2015, Henninger et al., 2016, Chen et al., 2018, Chuckowree et al., 2018). In the current study we found, in the internal capsule, that mTBI induced significantly higher axonal degeneration and more axonal fragments in both adult and aged cohrts at both 1 week and 4 weeks post injury. In contrast, mTBI increased axonal degeneration and axonal fragments at 1 week post injury and resolved at 4 weeks post injury. This finding suggests that the axonal pathology in the internal capsule was time and age dependent, with both short-term and long-term existing in adult and aged mice but only short-term onset in young mice, which may underlie the long-lasting functional deficits observed in old patients and eariler recovery in young patients (Pendlebury et al., 1999, Kinnunen et al., 2011, Li et al., 2019). Previous studies have observed exacerbated white matter loss in adult but not in adolescent mice at 3 months after repetitive mTBI (Mannix et al., 2017). In the current study, we found the axonal changes in the internal capsule at 4 weeks post injury after mild injury were only observed in adult and aged brain-injured mice but not in young mice. This suggests age dependent axonal degeneration after mTBI. Moreover, it may also suggest the young brain is resilient in regards to axonal degeneration or the axonal degeneration occurs earlier. Previous studies have observed aged brains had higher rates of axonal degeneration in the corpus callosum compared to young brains at acute and chronic time points after rmTBI (Ferguson et al., 2017b, Mouzon et al., 2018b). Similarly in the current study, we found the axonal changes in the internal capsule were higher in the aged injured brains compared to young injured brains at 4 weeks post-injury, which may suggest the vulnerability of aged brains to injury, and specifically axonal pathology.

Previous studies suggest higher astrocytic and microglial activation may play an important role in promoting axonal damage in the injured brains, as higher inflammation may result in a delay in the clearance of axonal degeneration debris (Kotter et al., 2005, Zhao et al., 2006). Our study however found that the astrocyte and microglia activation after injury was highest at 1 week post injury and 4 weeks post injury respectively, whereas the axonal degeneration occurred at both 1 week and 4 weeks post-injury. This discrepancy in timing suggests that glial activation does not necessarily correlate with axonal pathology, a view supported by Hilla's study (Hilla et al., 2017). Pathological changes in the internal capsule have been linked to functional deficits and poor recovery as well as quality of life (Pendlebury et al., 1999, Lee et al., 2000, Blasi et al., 2015, Hyett et al., 2018). The observed age dependent axonal degeneration in our study may underlie these age relevant outcomes following mTBI. The vulnerability of the aged brain to axonal pathology may due to the thinner neocortex, which may increase proximity between the site of impact and the underlying white matter in turn.

In summary, this data supports previous investigations reporting that the aged brain is characterized by a thinner cortex, increased glial activation as well as pronounced axonal degeneration in the internal capsule. Furthermore, we found axonal degeneration and microglial activation after mTBI was age and time dependent, with axonal degeneration lasting at both short-term and long-term in adult and aged but resolved at later timepoint in young brain injured mice, and microglial activation observed in adult but not in young or aged cohorts after mTBI.

4. THE THERAPEUTIC POTENTIAL OF THE MICROTUBULE STABILIZING AGENT EPOTHILONE D FOLLOWING MILD TRAUMATIC BRAIN INJURY

4.1. INTRODUCTION

mTBI results in substantial costs at individual and societal levels, with estimated total expenditures of 1.2 million spent on mTBI (Gerberding, 2003, Faul et al., 2010, Dewan et al., 2018). Additionally, there is no effective cure. mTBI does not cause overt morphological changes to the brain, however, it can set in motion an insidious cascade of neural alterations manifesting in ongoing and long-term impairments in neurological and cognitive function, as well as personality and behavioural changes (Eme, 2017, Shin et al., 2017, McInnes et al., 2017, Eman Abdulle and van der Naalt, 2019, Lai et al., 2019, Richey et al., 2019, Sun et al., 2019). mTBI induces widespread axonal impairments (including axonal swelling and degeneration) in the cortex and particularly in the white matter tracts as well as somato-dendritic compartment changes such as soma atrophy, dendritic complexity reduction and decreased dendritic spine density. Glial activation following mTBI has also been observed (Dikranian et al., 2008, Spain et al., 2010, Gao and Chen, 2011, Greer et al., 2011, Liu et al., 2013, Katz et al., 2015, Henninger et al., 2016, Winston et al., 2016, Chen et al., 2018, Rodriguez-Grande et al., 2018, Zhao et al., 2018, Clement et al., 2019, Powell et al., 2019).

Microtubules (MT) serve as the major railways for organelles and other cargoes transported within axons and dendrites. Appropriate MT function underlies the growth and maintenance of the axon, as well as the development and plasticity of the dendritic arbors (Conde and Cáceres, 2009). Microtubules are also an important constituent of non-neuronal cells in the brain. In oligodendrocyte, morphologically, microtubules can be classified into radial microtubules which are found along branched processes extending toward the axon, and lamellar microtubules which are distal microtubules mediating vesicular trafficking into inner myelin layers (Snaidero et al., 2014). Astrocyte processes contain primarily microtubules at birth, but these are entirely replaced by intermediate filaments during maturation (Peters and Vaughn, 1967). Microtubules in astrocyte can undergo extension, retraction and branching events (Eom et al., 2011). Microglia are highly dynamic, extending and retracting their processes. It has been observed that the major, more stable, processes contain microtubules while minor processes contain actin rich filopodia with dynamic processes (Bernier et al., 2019). MT disruption in neurons has been widely observed following brain and spinal cord injuries (Brunden et al., 2012, Tang-Schomer et al., 2012, Baas, 2014, Brizuela et al., 2015). Therefore, manipulating microtubules could possibly provide a novel therapy in these injuries (Brunden et al., 2012, Baas, 2014, Dent, 2017). Traditionally, microtubule stabilizing agents

including taxanes and epothilones have been widely used to inhibit cancer cell growth at high dosage (Zhao et al., 2009). Compelling experimental studies from brain injury, spinal cord injury, and neurodegenerative disease models have found that when used at low dosage, these agents could have a range of additional beneficial outcomes, including promoting axon regeneration, decreasing axon degeneration, increasing dendritic spine density, dampening glial responses, enhancing adaptive neural alterations and improving cognitive and behavioural outcomes (Brunden et al., 2012, Hellal et al., 2011, Sengottuvel et al., 2011, Hur and Lee, 2014, Popovich et al., 2014, Brizuela et al., 2015, Cross et al., 2015, Ruschel et al., 2015, Jang et al., 2016, Penazzi et al., 2016, Yap et al., 2017, Chuckowree et al., 2018, Ruschel and Bradke, 2018, Sandner et al., 2018, Xiong et al., 2019).

The mechanisms of epothilones include preventing microtubules depolymerization and promoting the tubulin polymerization to microtubules (Altmann et al., 2000, Chen et al., 2008). In regard to the CNS, epothilones are able to cross the blood brain barrier, have higher solubility, and have been shown to remain in the CNS for several days without any observable side effects (Andrieux et al., 2006, Brunden et al., 2011). Preclinical studies have demonstrated that the MT stabilizing agent epothilone D may be beneficial to treat brain injury, spinal cord injury, and neurodegenerative diseases (Brunden et al., 2010, Barten et al., 2012, Zhang et al., 2012, Cartelli et al., 2013, Fan et al., 2014, Brizuela et al., 2015, Ballatore et al., 2016, Penazzi et al., 2016, Yap et al., 2017, Chuckowree et al., 2018, Ruschel and Bradke, 2018, Sandner et al., 2018, Xiong et al., 2019). Our recent *in vivo* and *in vitro* studies have demonstrated epothilone D treatment significantly improved MT stability, reduced axonal fragmentation, and increased mushroom spine density following mild injury (Brizuela et al., 2015, Yap et al., 2017, Chuckowree et al., 2018). Another *in vitro* study showed that epothilone B could be both beneficial and detrimental to axonal growth depending on the age and type of a neuron (Jang et al., 2016). Thus, in order to further investigate the therapeutical potential of epothilone D and to determine if the drug effect is age dependent, we explored the short-term and long-term effects of peripherally administered epothilone D following a single mild LFPI in both young and adult mice.

4.2. MATERIALS AND METHODS

Studies were performed on young (1.5 months) and adult (3 months) Thy1-YFPH transgenic male mice. Mice were randomized to the injury group (n=48 young, and n=48 adult) and the sham group (n=48 young, and n=48 adult). Lateral fluid percussion injury (LFPI) of mild severity (1.29±0.11 atmospheres) was delivered to the ‘inhjury’ cohort. At 24 hours following the injury/sham-operation, animals were administered with either epothilone D (EpoD)

(2mg/kg, i.p; Abcam, ab143616, Cambridge, UK; n=24 injury and n=24 sham-operated) or dimethyl sulfoxide (DMSO) (vehicle control; i.p; Sigma, RNBD0643, Missouri, USA; n=24 injury and n=24 sham-operated), and then with weekly injection of same dosage of epothilone D or equivalent volume of DMSO (Fig 4.1). The specific dose of EpoD was chosen as there was in the absence of major side effect (Scott et al., 2008), and the potential effectiveness of this dosage has been observed among mTBI and ALS animal models (Chuckowree et al., 2018, Clark et al., 2018). However, using various doses could be informative in future studies. Additionally, the rationale for choosing the 24 hrs delay in drug injection is allow for parameters of clinical relevance. For example, it takes a while for injured patients with transportation, clinical assessment and diagnosis before the drug treatment. Half of mice were perfused at 1 week post injury (n=6 young FPI EpoD 1wk, n=6 young FPI DMSO 1wk, n=6 young sham EpoD 1wk, n=6 young sham DMSO 1wk, n=6 adult FPI EpoD 1wk, n=6 adult FPI DMSO 1wk, n=6 adult sham EpoD 1wk, n=6 adult sham DMSO 1wk) and the other half were perfused at 4 weeks post injury (n=6 young FPI EpoD 4wk, n=6 young FPI DMSO 4wk, n=6 young sham EpoD 4wk, n=6 young sham DMSO 4wk, n=6 adult FPI EpoD 4wk, n=6 adult FPI DMSO 4wk, n=6 adult sham EpoD 4wk, n=6 adult sham DMSO 4wk). For the 4 weeks cohort, mice received the first dose at 24 hr after injury then once a week till 4 weeks post injury. Details regarding immunohistochemistry protocols can be found in Chapter 2. Statistically significant differences are noted on graphs where present.

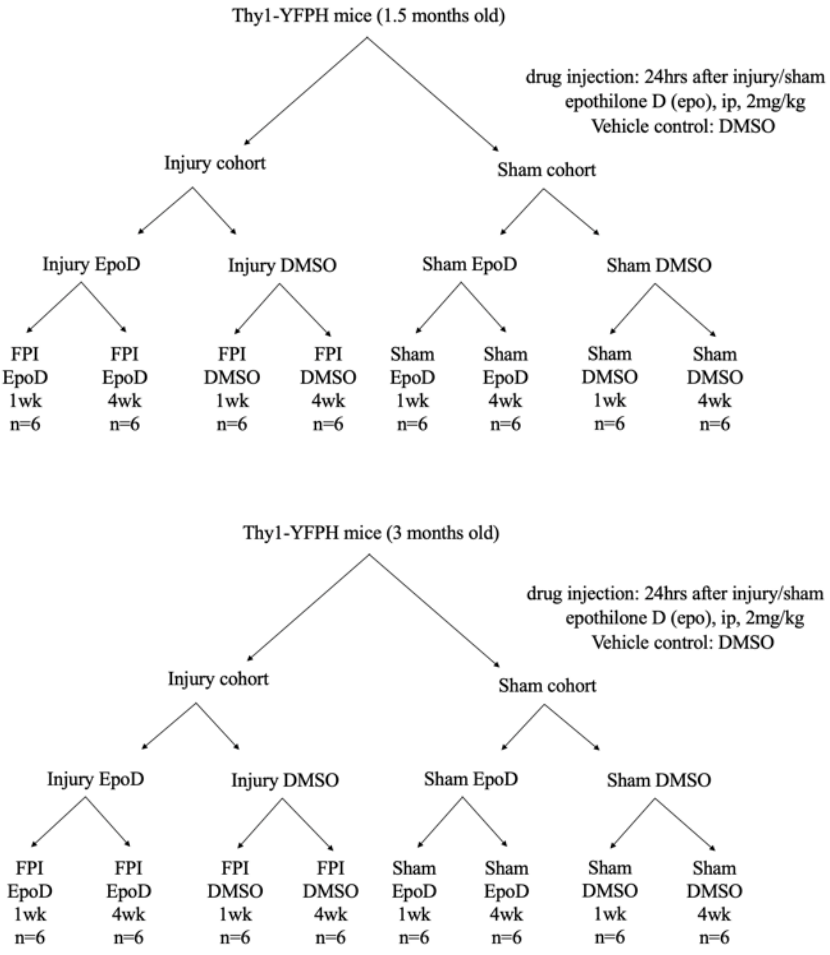

Figure 4.1 Experimental flow charts.

A lateral fluid percussion injury/sham procedure was conducted in both young (1.5 months old) and adult (3 months old) Thy1-YPFH mice. At 24 hours post injury, all the mice were injected with either EpoD or DMSO, and then with weekly injections of same dosage of EpoD or equivalent volume of DMSO. Animals were perfused at either 1 week post injury or 4 weeks post injury.

4.3. RESULTS

4.3.1. EpoD did not affect cortical architecture in young and adult mice

The injury pulse used in young (1.5 months) injured mice was comparable to that given to adult (3 months) injured mice (Fig 4.2 A; p=0.4456). LFPI was followed by a short interval of apnoea (Fig 4.2 B) in animals at 1.5 months and 3 months of age at injury, additionally, the duration of apnoea was significantly longer in young (1.5 months) brain-injured mice in comparison to adult (3 months) brain-injured mice (p=0.0036). In regard to the delayed righting reflex time, a Two-way ANOVA revealed no significant interaction between injury and age at injury (Fig 4.2 C; $F_{1,91}$ =8.113, p=0.0054). The righting reflex time however was significantly increased following LFPI compared to shams in animals injured at the age of 1.5 months (p<0.0001) and 3 months (p<0.0001). As to the cortical thickness, an ordinary Three-way ANOVA revealed no significant interaction between week post injury and age at the time of injury in sham-operated mice (Fig 4.2 E; $F_{1,39}$ =0.01270, p=0.9108) and brain injured mice (F 4.2 F; $F_{1,39}$ =1.231, p=0.2741). Similarly for the YFP positive cell density and the cell soma size, there was no significant interaction between week post injury and age at the time of injury in sham-operated mice (Fig 4.2 G; $F_{1,39}$ =0.001166, p=0.9729; Fig 4.2 I; $F_{1,39}$ =0.6855, p=0.4127, respectively) and brain-injured mice (Fig 4.2 H; $F_{1,39}$ =0.2087, p=0.6503; Fig 4.2 J; $F_{1,39}$ =0.0359, p=0.8507, respectively).

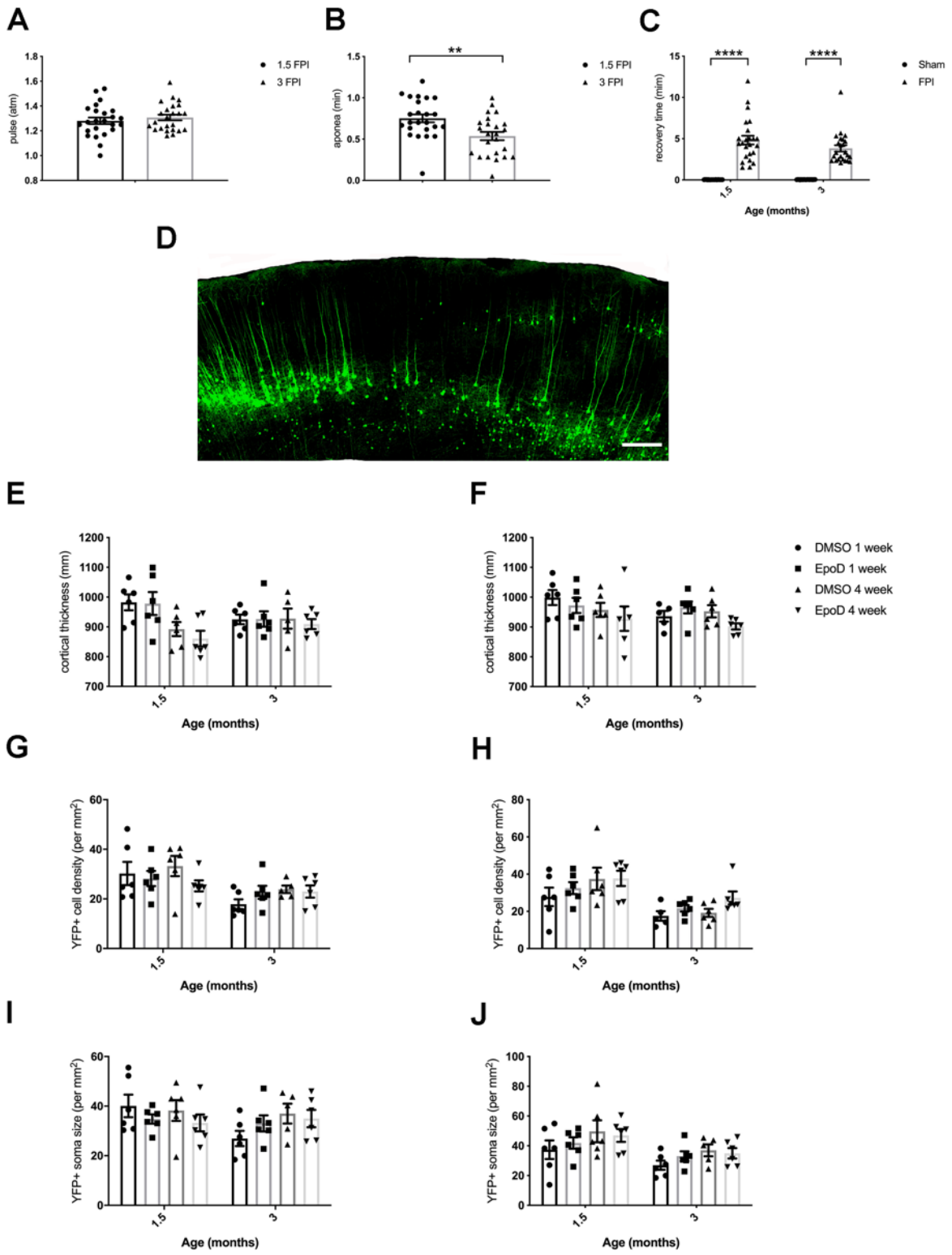

Figure 4.2. EpoD did not influence neuronal health in young and adult mice.

The injury pulse was not different between young (1.5 months) and adult (3 months) brain-injured cohorts (A). LFPI caused a short period of apnoea in animals of 1.5 months and 3 months old, and the apnoea was longer in brain-injured animals at the age of 1.5 months

*compared to 3 months (B). LFPI induced a significant increase in righting reflex time in both 1.5 months and 3 months old mice, and there was no significant difference between injured mice at 1.5 months old and at 3 months old (C). Representative image of LFPI in an young Thy1-YFPH mouse brain with EpoD treatment (D). Cortical thickness did not differ between EpoD treatment and DMSO treatment in sham-operated mice (E) and brain-injured mice (F) at the age of 1.5 months and 3 months at injury, at either 1 week or 4 weeks post injury. The YFP positive cell density in the neocortex was not different between EpoD treatment and DMSO treatment in sham-operated mice (G) and brain-injured mice (H) at the age of 1.5 months and 3 months at injury, at either 1 week or 4 weeks post injury. The YFP positive cell soma size in the neocortex was not different between EpoD treatment and DMSO treatment in sham-operated (I) and brain-injured mice (J) at the age of 1.5 months and 3 months at injury at either 1 week or 4 weeks post injury. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's tests **p<0.01, ****p<0.0001. Scale bar=200um.*

4.3.2. EpoD treatment did not alter the response of gliosis in young and adult mice

The effect of EpoD on GFAP immunoreactivity was investigated within the neocortical region of the injury site (experimental details can be found in Chapter 2) in sham-operated mice (Fig 4.3 C) and brain-injured mice (Fig 4.3 D). An ordinary Three-way ANOVA revealed no significant interaction between week post injury and age at injury in sham-operated mice ($F_{1,39}$ =2.513, p=0.1210) and brain-injured mice ($F_{1,39}$ =0.2652, p=0.6095). However, astrocyte activation in brain-injured EpoD treated mice was significantly resolved at 4 weeks post injury in comparison to 1 week post injury with either DMSO or EpoD treatment in mice at the age of 1.5 months (p=0.0115, p=0.0083, respectively) and at the age of 3 months (p=0.0011, p=0.0007, respectively). The effect of EpoD on Iba1 immunoreactivity was investigated within the same region of analysis as GFAP (experimental details can be found in Chapter 2) in sham-operated mice (Fig 4.3 E) and brain injured mice (Fig 4.3 F). An ordinary Three-way ANOVA revealed no significant interaction between week post injury and age at injury in sham-operated mice and brain-injured mice ($F_{1,40}$ =0.2913, p=0.5924; $F_{1,39}$ =0.4788, p=0.4930, respectively). However, microglial activation was significantly resolved at 4 weeks post injury in comparison to 1 week post injury with DMSO treatment in animals at 3 months old at injury (p=0.0059).

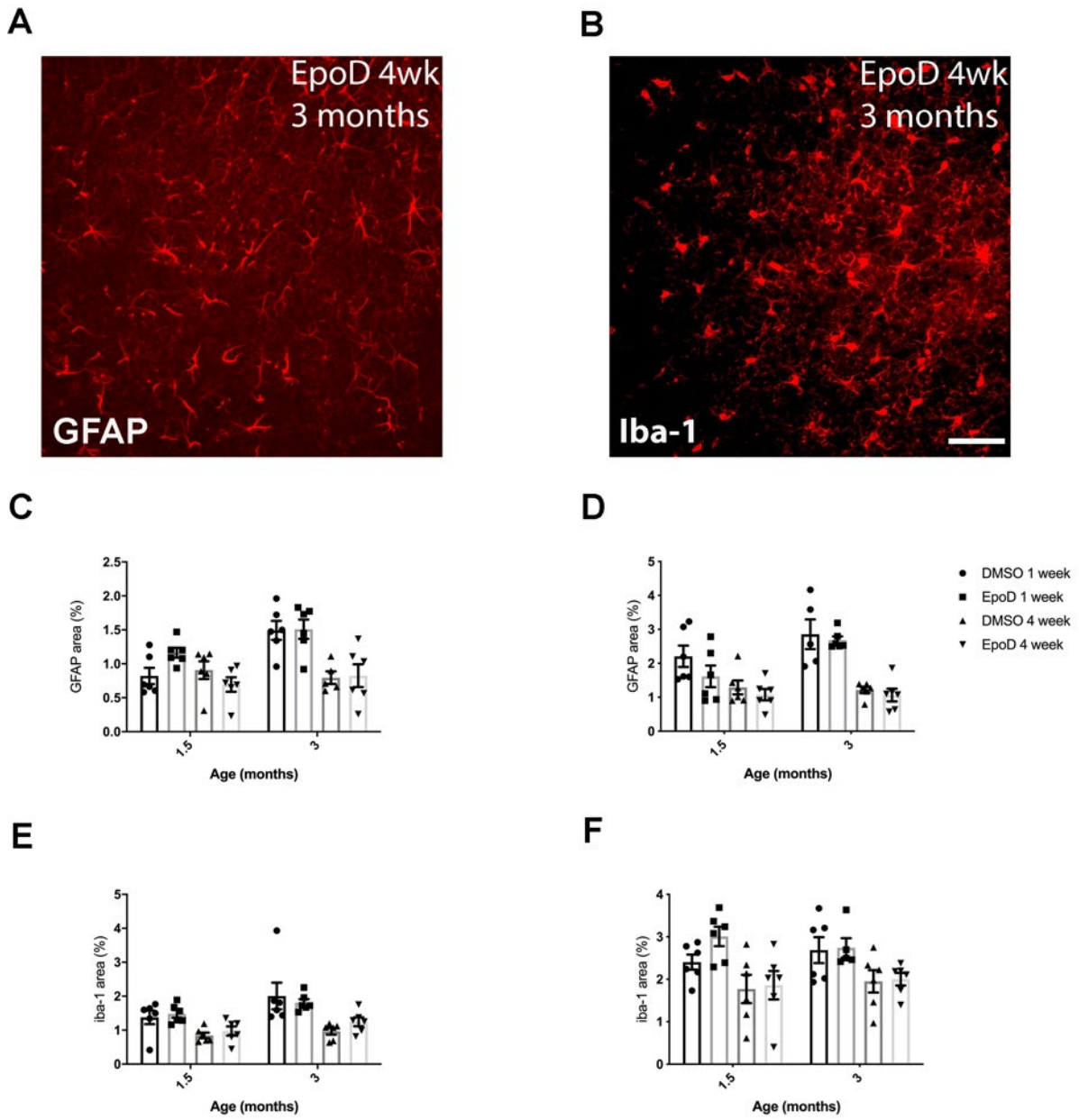

Figure 4.3. EpoD did not influence astrocytic or microglial activation in both young and adult mice.

Representative image of mTBI on astrocyte (A) and microglia (B) in the neocortex. In the injured neocortex, the percentage of GFAP immunoreactivity was not significantly influenced by EpoD treatment compared to DMSO treatment in either sham-operated mice (C) or brain-injured mice (D) at the age of 1.5 months and 3 months at injury. Similarly, the percentage of Iba1 immunoreactivity was not significantly affected by EpoD treatment in comparison to DMSO treatment in either sham-operated mice (E) or brain-injured mice (F) at the age of 1.5 months and 3 months at injury. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests. Scale bar=50um.

4.3.3. EpoD exacerbated axonal degeneration after mTBI in the internal capsule in adult mice but was ineffective in young mice

The effect of EpoD on axonal degeneration in the IC over time was investigated in 1.5 and 3 months old mice injured and sham mice (Fig 4.4 A). In regard to the total amount of axonal degeneration, an ordinary Three-way ANOVA revealed no significant interaction between week post injury and age at injury (Fig 4.4 B; $F_{1,31}$ =2.131, p=0.1544). However there was a significant interaction between age at injury and treatment ($F_{1,31}$ =32.01, p<0.0001). Tukey's multiple comparisons tests identified multiple significant differences. Axonal degeneration was significantly increased at 4 weeks post injury with EpoD treatment in comparison to DMSO treatment in animals injured at 3 months old (p=0.0026). Furthermore, the axonal degeneration at 4 weeks post injury with EpoD treatment in 3 months old mice was significantly pronounced in comparison to axonal degeneration at 4 weeks post injury with EpoD treatment in 1.5 months old (p<0.0001), indicating that the effect of EpoD on axonal degeneration worsened over time.

As to the relative number of axonal fragments (Fig 4.4 C). An ordinary Three-way ANOVA revealed no interaction between week post injury and age at injury ($F_{1,30}$ =1.6460, p=0.2093). However there was a significant interaction between age at injury and treatment ($F_{1,30}$ =14.98, p=0.0005). Tukey's multiple comparisons tests identified multiple significant differences. There was a significant increase in the number of axonal fragments at 4 weeks post injury with EpoD treatment in animals injured at 3 months of age compared to 1.5 months of age (p=0.00071).

In regard to the size of the axonal fragments following EpoD treatment (Fig 4.4 D). An ordinary Three-way ANOVA revealed no interaction between week post injury and age at injury ($F_{1,32}$ =0.0384, p=0.8458). However there was a significant interaction between age at injury and treatment ($F_{1,32}$ =5.061, p=0.0315). Tukey's multiple comparisons tests identified no significant differences.

Together these data suggests EpoD was ineffective on axonal degeneration in young brain injured mice, but it significantly increased axonal degeneration in adult brain injured mice at 4 weeks post injury.

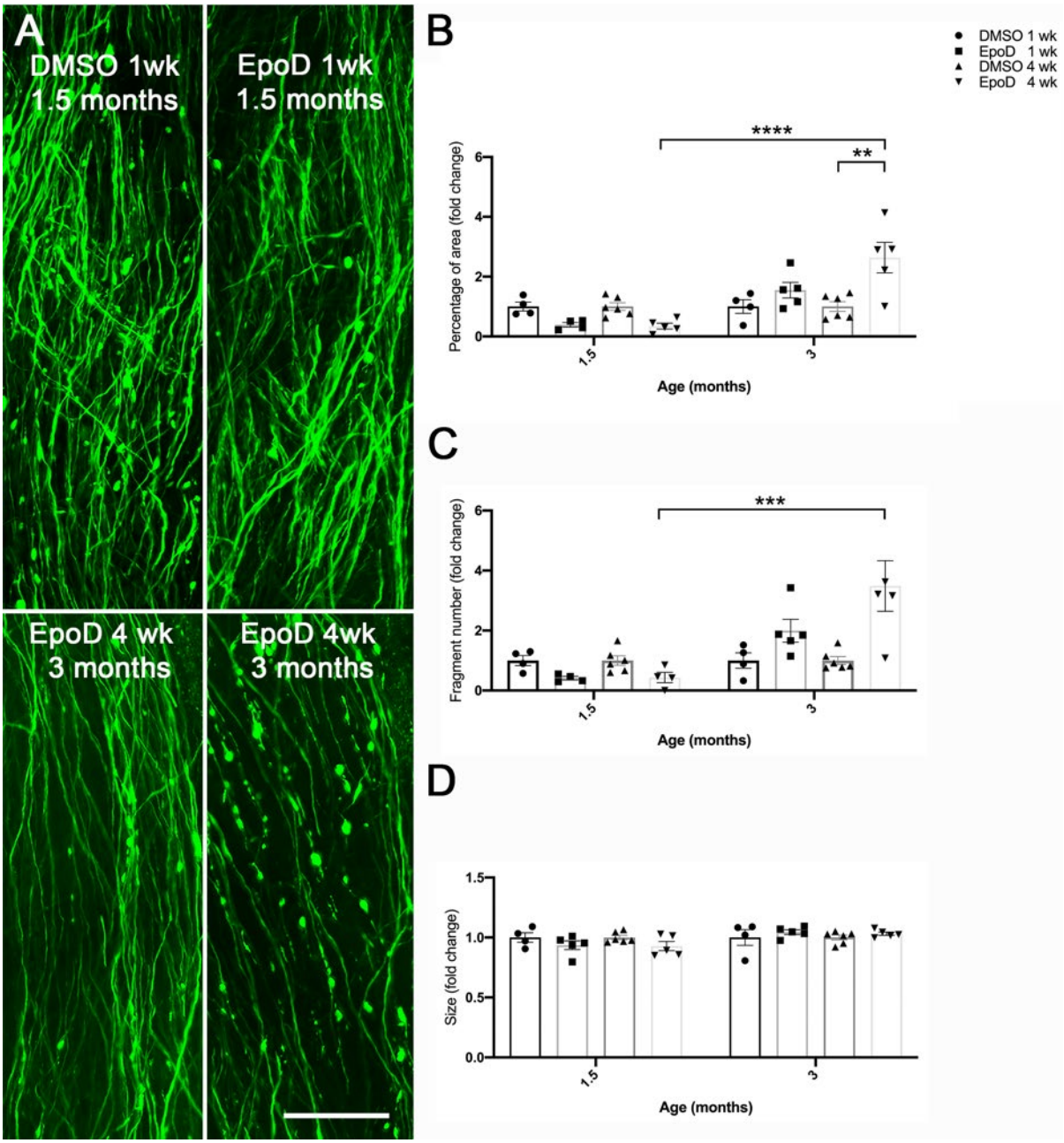

Figure 4.4. EpoD exacerbated axonal degeneration after mTBI in the internal capsule in adult mice and was ineffective in young mice.

Representative images of mTBI on axons in the internal capsule of the Thy1-YFPH transgenic mouse brain (A). The total amount of axon degeneration was significantly increased by EpoD treatment compared to DMSO treatment at 4 weeks post injury in brain-injured animals at 3 months old, the axonal degeneration at 4 weeks post injury in brain injured animals at 3 months old at injury was significantly higher in comparison to 4 weeks post injury in brain injured animals at 1.5 months old at injury (B). The number of axon fragments at 4 weeks post injury in EpoD treated mice was significantly higher in animals at 3 months old at injury in comparison to 1.5 months at injury (C). The size of axon fragments

*did not change after EpoD treatment compared to DMSO treatment in brain injured animals at either 1.5 months or 3 months old at injury (D). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests **p<0.01, ***p<0.001, ****p<0.0001. Scale bar=40um.*

4.3.4. EpoD did not affect the total spine density or density of sub-classes of spines in young and adult mice

In the current study, there was no significant difference in total spine density in EpoD treated animals in comparison to DMSO treated sham-operated animals (Fig 4.5 A; $F_{1,40}$ =0.01585, *p*=0.9004) and brain-injured animals (Fig 4.5 B; $F_{1,39}$ =0.0110, *p*=0.9172) at either 1.5 months or 3 months old cohorts, at either 1 week or 4 weeks post injury. Similarly for each individual morphological subtype, there were no significant interactions between spine density, age at injury of time post injury for thin spines in sham-operated mice (Fig 4.5 C; $F_{1,39}$ =3.062, *p*=0.0880) and brain-injured mice (Fig 4.5 D; $F_{1,39}$ =0.8377, *p*=0.3657), stubby spines in sham-operated mice (Fig 4.5 E; $F_{1,39}$ =0.1668, *p*=0.6852) and brain-injured mice (Fig 4.5 F; $F_{1,39}$ =0.0218, *p*=0.8833), or mushroom spines in sham-operated mice (Fig 4.5 G; $F_{1,39}$ =0.03692, *p*=0.8486) and brain-injured mice (Fig 4.5 H; $F_{1,39}$ =1.131, *p*=0.2940). Overall, EpoD did not have any significant influence on the density of total spines or sub-classes of spines.

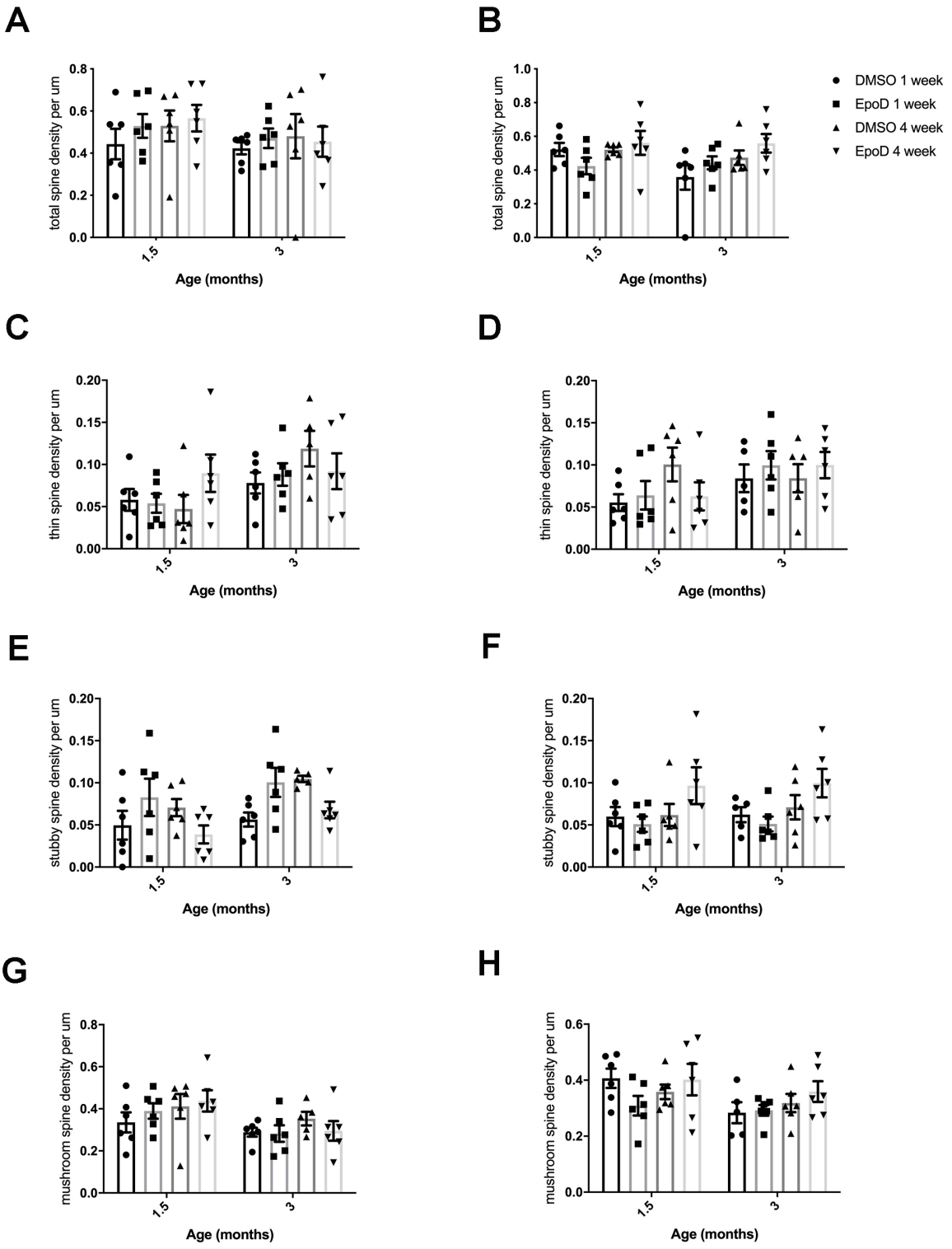

Figure 4.5. EpoD did not affect the density of total spines or the density of sub-classes of spines in young and adult mice.

Total spines density was not influenced by EpoD treatment compared to DMSO treatment in either sham-operated mice (A) or brain-injured mice (B) at the age of 1.5 months and 3 months at injury. Likewise, as to each individual subtype, the density of thin spines (C and

D), stubby spines (E and F), or mushroom spines (G and H) was not affected by EpoD treatment in comparison to DMSO treatment in sham-operated mice or brain-injured mice at the age of 1.5 months and 3 months at injury. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's multiple comparisons tests.

4.4. DISCUSSION

Despite limited overt morphological changes to the brain in mTBI patients, compelling evidence suggests neural circuitry impairments play an important role in the alterations of neurological function, including physical, psychosocial, neurosensory and cognitive function (McMahon et al., 2014, Wang and Li, 2016, Wilde et al., 2016, De Koning et al., 2017). The cellular changes contributing to mTBI deficits are less well studied, and currently there are no effective treatment to rescue these changes. Previous *in vitro* studies in our lab using cultured primary neurons have shown the potential of axonal sprout promotion and reduction of injury related axonal fragments by epothilone D treatment (Brizuela et al., 2015, Yap et al., 2017). Additionally, another *in vivo* study in our lab has further suggested the therapeutic potential of epothilone D in mTBI by reporting an increase in the density of mushroom spines after a single dose of epothilone D immediately following mTBI in adult mice (Chuckowree et al., 2018). In the present study, we further investigated therapeutic potential of the drug by applying multiple low doses of epothilone D at 24 hours after mTBI in both young (1.5 months) and adult (3 months) mice. Consistent with previous studies, we observed mTBI induced apnoea and increased loss of consciousness in both young (1.5 months) and adult (3 months) mice. Additionally, we further supported findings that intraperitoneally injected epothilone D was able to cross the blood brain barrier, and both single and multiple doses of epothilone D in our study did not trigger gross neural population and glial activation changes. Importantly, we found that although there wasn't any effect on axonal degeneration following epothilone D treatment in young cohorts, epothilone D significantly exacerbated axonal degeneration in adult brain-injured mice, potentially suggesting the need for age dependent drug therapy for mTBI patients.

Similar to previous studies (Smith and Meaney, 2000, Spain et al., 2010, Wang and Ma, 2010, Ekmark-Lewén et al., 2013, Smith et al., 2013a, Hill et al., 2016) and results in Chapter 3, in the present study, we found that the mild injury did not alter architecture in the cortex, supported by in the absence of overt neuron loss, neuronal atrophy and cortical loss at both 1 week and 4 weeks after injury. Additionally, LFPI caused a short duration of apnoea and righting reflex both in young (1.5 months) and adult (3 months) animals. Similar to findings in Chapter 3, the righting reflex time was not different between young and adult mice, however, the duration of apnoea was significantly longer in young mice compared to adult mice. This is similar to Rowe and colleagues findings, in which mild midline FPI induced duration of apnoea was significantly longer in juvenile (17 days old) and adolescent (35 days old) rats (Rowe et al., 2018). Thus, even without difference in righting reflex time between young and adult mice following mTBI, the duration of apnoea was longer in young mice compared to adult mice.

In the present study, we further confirmed that both single and multiple doses of epothilone D did not cause overt morphological changes to cortical pyramidal neurons in both young (1.5 months) and adult (3 months) mice, supported by no significant changes in the cortical thickness, the YFP positive cell density and the cell soma size (Chuckowree et al., 2018). Furthermore, similarly to previous studies (Hellal et al., 2011, Ruschel et al., 2015, Chuckowree et al., 2018), we showed that injury induced astrocytic activation was not affected by epothilone D treatment in both adult mice and young mice. Some previous studies (Popovich et al., 2014, Ruschel et al., 2015) have suggested microtubule stabilizing agent drugs can affect astrocyte activation, however, Popovich and colleagues used taxol, and Ruschel and colleagues applied epothilone B in a model of spinal cord injury. Therefore, the discrepancy may be due to variance in drugs and types of injury. The influence of epothilone D on microglial activation is less well studied. Our findings suggest that microglial activation is not affected by both single and multiple doses of epothilone D in both young and adult mice after mTBI. A previous study from our laboratory (Clark et al., 2018) has reported that epothilone D injection (2 mg/kg at 5 days intervals) significantly increased microglial activation in the end stage of an amyotrophic lateral sclerosis (ALS) mouse model, suggesting epothilone D may have an effect on microglial activation at longer timepoints and in different disease contexts. Thus, further studies investigating chronic effects of epothilone D after mTBI are warranted.

Microtubule stabilizing agent drugs have been reported with promising results in spinal cord injuries, where they have been reported to promote axonal regeneration and improve walking function after spinal cord injury. The underlying mechanisms may be relevant to reducing fibrotic scarring and promoting regrowth of injured spinal cord axons following microtubule stabilizing agent drugs delivery (Hellal et al., 2011, Ruschel et al., 2015, Ruschel and Bradke, 2018). epothilone D has been reported to stabilize microtubules, promote of neurite outgrowth, and reduce injury-related axonal fragments in cultured neurons (Brizuela et al., 2015, Yap et al., 2017). In mouse models of neurodegenerative diseases, low doses of epothilone D significantly increased MT density and axonal integrity (Brunden et al., 2010). Additionally, epothilone D treatment was also found to reduce axonal dystrophy and increase axonal MT density in a Tau transgenic mouse model (Zhang et al., 2012). Furthermore, it has been reported that epothilone D was able to prevent Aβ-induced axon and cell loss in Tau transgenic mouse model (Nishioka et al., 2019). In a cerebral ischemic model, epothilone D has been found to alleviate microtubule disruption and CA1 neuronal death (Xiong et al., 2019). In the present study, we found epothilone D did not affect injury induced axonal degeneration at 1 week post-injury in adult brain-injured mice, which is similar to our previous study (Chuckowree et al., 2018). Furthermore, we also validated the ineffectiveness of

epothilone D in improving measures of axonal degeneration at both 1 week and 4 weeks post-injury in young brain-injured mice. However, we found multipe doses of epothilone D (4 injections at 1 week interval) significantly exacerbated axonal degeneration in the internal capsule compared to DMSO treatment in adult brain-injured mice. These findings suggest the effect of epothilone D on axonal degeneration following mTBI was age dependent. The age dependent effect of the drug has been reported with an epothilone D analog, epothilone B. It has been observed that epothilone B promoted axonal growth of young dorsal root ganglion (DRG) and cortical neurons, while the same concentration drastically prevented axon growth of adult sensory neurons and caused severe toxicity in adult DRG neurons (Jang et al., 2016). It is possible that the proportion of stable microtubules is different depending on neuron age, as most stable microtubules in neurons play an important role in maintaining the structural integrity, while dynamic microtubules provides the basis for neural plasticity and structural modifications in response to stimuli. Additionally, it is plausible that a neuron of a particular age harbors MT of unique biochemical and biophysical properties that control MT stability (Jang et al., 2016).

It has been reported that a subnanomolar concentrations of epothilone D could completely reverse Aβ-induced spine loss and also increase thin spin density in an *in vitro* study (Penazzi et al., 2016). Additionally, it has been also reported that a single low dose of epothilone D administered immediately after mild injury significantly increased the density of mushroom spines in a LFPI mouse model (Chuckowree et al., 2018). However, in the current study, we did not observe any changes in the overall density or sub-class density of spines following the drug treatment in either young or adult mice. The discrepancy may due to different timepoints of drug injection - Chuckowree and colleagues injected the drug immediately after injury, while in the current study we delivered the drug at 24 hours after injury, this may suggest the effect of epothilone D on spine density is dependent on the delivery timepoints.

In summary, this data supports previous investigations reporting that epothilone D did not affect neuronal density and glial activation in the neocortex in either young or adult mice. Furthermore, we showed that age dependent effects of epothilone D on injury related axonal degeneration in the internal capsule with ineffectiveness in young animals and increased axonal degeneration in adult animals. Our findings may support the need for consideration of age when designing drug therapy for mTBI.

5. THE INFLUENCE OF SEX AND AN ADDITIONAL INJURY ON THE CELLULAR RESPONSES TO MILD TRAUMATIC BRAIN INJURY

5.1. INTRODUCTION

mTBI has been recognized as a silent epidemic, however, it is also characterized by a disturbance in brain function with neurological symptoms (Carroll et al., 2004, Hossain et al., 2019). The majority of TBI studies are performed in male populations and/or using male animal models, partially due to the overall higher TBI incidence in males than females (Hillbom and Holm, 1986, Hirtz et al., 2007, Taylor et al., 2017), with females sustaining approximately only a third of TBIs resulting in hospitalisation (Faul et al., 2010, Saverino et al., 2016). It is, however, also reported that females typically have a high prevalence of mild TBI due to domestic violence, and as a result of high participation in health care occupations (Chang et al., 2014, Covassin et al., 2016, Colantonio, 2016, Lutgendorf, 2019), while males are more likely to suffer a moderate to severe brain injury resulting from high-risk activities, such as professional contact sports, the construction industry, or military occupations (Diamond et al., 2007, Cohen, 2013, Chang et al., 2014, Raukar et al., 2014, Chang et al., 2015). In sport-related injuries as well as former military personnel, repetitive mTBI has been widely observed. While behavioural and cognitive deficits resulting from a single mTBI may resolve over time after injury, clinical studies have shown additional injuries may increase the susceptibility to long lasting functional impairments (Cheng et al., 2014a, Ferguson et al., 2017a, Gunter et al., 2018, McAllister and Wall, 2018, Merritt et al., 2018, Misquitta et al., 2018, Kontos et al., 2019, Ruiter et al., 2019). Furthermore, repetitive injuries may be associated with increased risk of the developing later-life neurodegenerative diseases (Lehman et al., 2012) including Alzheimer's disease (AD) and chronic traumatic encephalopathy (CTE) (Guo et al., 2000, Plassman et al., 2000, Fleminger et al., 2003, Guskiewicz et al., 2005, McCrory et al., 2013, McKee et al., 2013, Smith et al., 2013b, Baugh et al., 2014, Nordström et al., 2014, Abrahams et al., 2019).

Sex dependent outcomes following TBI have been reported in a number of studies, but with mixed findings. In some clinical studies females showed worse outcomes across multiple measures, including duration of post-traumatic amnesia, memory impairment, insomnia, and mortality (Farace and Alves, 2000, Biswas et al., 2017), as well as self-reported headaches, dizziness, and depression (Colantonio et al., 2010, Merritt et al., 2019, Yue et al., 2019). However, other studies report no significant difference in working memory performance between female and male patients after mTBI (Hsu et al., 2015). In pre-clinical studies, some studies have reported better performance in female animals compared to male animals after

TBI (Dick, 2009, Bazarian et al., 2010, Frommer et al., 2011, Laker, 2011, Covassin and Bay, 2012, Velosky et al., 2017, Clevenger et al., 2018). However, in other investigations no sex dependent differences in behavioural or pathological outcome in young mice after repetitive mild injures were observed, whilst others report that female mice showed worse motor outcome after repetitive mild injuries compared to male mice (Ferguson et al., 2017b).

Furthermore, sex related differences in inflammation responses after injury have also been reported. Späni and colleagues, report a significant increase in IL-6, TNFα, and C-C Motif Chemokine Ligand 2 (CCL2) in adult female mice compared to male mice after TBI, whereas the anti-inflammatory cytokine IL-10 was elevated in males but not in females (Späni et al., 2018). Sex related differences have also been reported for other pro-inflammatory factors. Both cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are increased at 24hr after TBI, but COX-2 is significantly elevated in males whereas iNOS is significantly increased in females compared to males (Gunther et al., 2015). Additionally, male pigs show a higher IL-6 increase at 4hr after TBI compared to female pigs receiving the same injury (Armstead et al., 2016).

To understand the pathological and behavioural changes following repetitive mTBI, experimental studies have tried to replicate the changes observed in repetitive mTBI patients. As with sex investigations, results from experimental studies are mixed, in which variance in numbers of injuries and interval between injuries may play a role. While some suggest shorter intervals between injuries are associated with exacerbated pathology, others found the interval between injuries did not affect long term pathological and behavioural outcomes (Cheng et al., 2014a, Selwyn et al., 2016, Multani et al., 2016, Ferguson et al., 2017a, Merritt et al., 2018). Thus, additional experimental studies are needed to better characterize the influence of the number of injuries sustained and the time interval between injuries on the pathological outcomes following repetitive mTBI.

Mixed clinical and experimental sex related findings after TBI highlight the importance of further investigating the influence of sex on outcomes following TBI. Those observed sex dependent behavioural and pathological changes after TBI may be partially attributed to the cellular and subcellular changes between sexes, in which, neuronal and glial reactions may play an important role (Conejo et al., 2005, Pesaresi et al., 2015, Villa et al., 2018). Furthermore, in order to identify potential therapeutic strategies to alleviate or prevent damage and deficits after repeated mTBI, the pathophysiological changes after repeated mTBI need further investigation. Thus, the final experimental chapter of this thesis presents two studies

investigating how the potential risk factors, sex and additional injury, influence the cellular and subcellular reactions after lateral fluid percussion in a mouse model.

5.2. MATERIALS AND METHODS

The influence of sex study was performed on adult (3 months) Thy1-YFPH transgenic male (same mice used in Chapter 3) and female mice. Female mice were randomized to the injury group (n=12) and sham group (n=12). Lateral fluid percussion injury (LFPI) of mild severity (1.34±0.12 atmospheres) was delivered to the injured mice. Half of the mice were perfused at 1 week post injury (n=6 female FPI 1wk and n=6 female sham 1wk) and the other half were perfused at 4 weeks post injury (n=6 female FPI 4wk and n=6 female sham 4wk).

The influence of an additional injury study was performed on adult (3 months) Thy1-YFPH transgenic male mice. The single brain injured mice were the same mice in Chapter 3. With regards to mice with repetitive mTBI, firstly, identical surgical procedures and lateral fluid percussion brain injury were performed (details can be found in Chapter 2), and then at 48hrs after the initial injury, mice underwent the same surgical procedures except for the absence of craniotomy, and then received the same lateral fluid percussion injury (details can be found in Chapter 2). The time interval of 48 hrs between mild CCI injuries has been used in adult mice to replicate repetitive brain injuries (Mouzon et al., 2012, Ojo et al., 2013). In the current study, 48 hrs injury interval was chosen as it is a temporal window during which the secondary injury is still existing and the mouse brain is susceptible to later injuries (Longhi et al., 2005). Additionally, this injury interval was selected in order to mimic injuries in patients, in which additional injuries are sustained prior to full recovery from the previous injury. Further investigations where this interval is both increased and decreased would likely reveal interesting insights, but were beyond the scope of this study. Finally, mice were perfused at either 1 week or 4 weeks post initial injury. Mice were randomized to 1 week (n=6) and 4 weeks (n=4) injured group. Details regarding immunohistochemistry protocols can be found in Chapter 2. Statistically significant differences are noted on graphs where present.

5.3. RESULTS

5.3.1. mTBI resulted in significant cortical thinning at 4 weeks post injury in female mice but not male mice

TBI in the mouse is considered 'mild' if the mortality rate after experimental injury is between 0 and 5%, a righting reflex occurs within 2 and 4 minutes and the loss of consciousness is less than 2 minutes (Morehead et al., 1994, Alder et al., 2011). In the present study, no mortality was observed, and the LFPI was associated with a short duration of apnoea (Fig 5.1 B). The duration of apnoea did not differ between male and female injured mice (p=0.8725). In regard to the righting reflex time (Fig 5.1 C), two-way ANOVA revealed no significant interaction between sex and injury ($F_{1,42}$ =0.1824, p=0.6715). However, as expected, the loss of consciousness was significantly increased in brain-injured mice in comparison to sham-operated mice in both males (p<0.0001) and females (p<0.0001). With regards to the measure of cortical thickness (Fig 5.1 D), an ordinary three-way ANOVA found a significant interaction between sex and the time post-injury ($F_{1,41}$ =4.773, p=0.0347). Tukey's multiple comparison tests found multiple significant differences. The cortical thickness was thinner in female sham-operated mice compared to male sham-operated mice at 1 week post injury (p=0.0383). Additionally, the cortical thickness in the cohort of female brain injured mice was significantly thinner compared to male brain injured mice at both 1 week and 4 weeks post injury (p=0.0005, p<0.0001, respectively). Furthermore, the injury resulted in a significantly decreased cortical thickness at 4 weeks post-injury compared to sham-operated mice in females (p=0.0004). In regard to the YFP positive cell density (Fig 5.1 E) and the cell soma size (Fig 5.1 F), an ordinary three-way ANOVA revealed no significant interaction between sex and the time post injury ($F_{1,41}$ =0.8087, p=0.3738; $F_{1,41}$ =0.9176, p=0.3437).

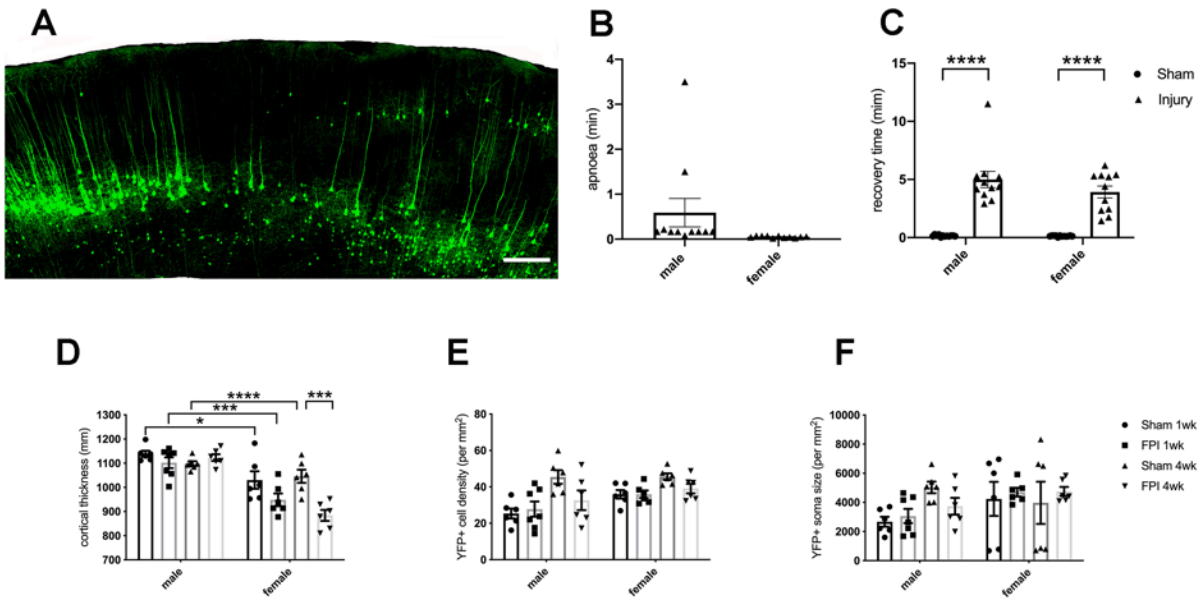

Figure 5.1. Cortical thickness was reduced in females at 4 weeks post injury compared to sham injured animals but was unchanged in males.

*Representative image of LFPI in an female Thy1-YFPH mouse brain (A). LFPI induced duration of apnoea did not differ between male and female brain-injured mice (B). LFPI induced loss of consciousness was not different between male and female brain-injured mice (C). The cortical thickness did not change after injury compared to shams in males, but was significantly decreased at 4 weeks post injury in female brain-injured mice compared to shams. Additionally, the cortical thickness in sham-operated and brain-injured mice was reduced in female compared to male mice at 1 week post injury. Furthermore, the cortical thickness in sham mice was thinner in female compared to male mice at 4 weeks post injury (D). The YFP positive cell density (E) and the cell soma size (F) in the cortex did not change at either 1 week or 4 weeks post mild injury compared to shams in both male and female mice. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's test *p<0.05, ***p<0.001, ****p<0.0001. Scale bar=200um.*

5.3.2. Glial response after mTBI is distinctive in male and female mice

The consequence of mTBI on GFAP immunoreactivity was investigated within the neocortical region of the injury site (experimental details can be found in Chapter 2). In regards to astrocyte activation (Fig 5.2 C), an ordinary three-way ANOVA revealed no significant interaction between sex and the time post injury ($F_{1,39}$ =0.8839, p=0.3529). Astrocytic activation was not significantly different between males and females in either sham operated or brain injured cohorts. Furthermore, astrocyte activation was significantly increased at 1 week post injury in brain-injured mice in comparison to sham-operated mice in females (p=0.0011). Overall, this data indicates that astrocyte activation was similar between males and females in both sham operated and brain injured cohorts, and the injury resulted in higher astrocytic activation only in females at 1 week post injury and resolved by 4 weeks post injury.

The consequence of mTBI on Iba1 immunoreactivity was investigated within the same region of analysis as GFAP (experimental details can be found in Chapter 2). As to microglial activation (Fig 5.2 D), an ordinary three-way ANOVA revealed no significant interaction between sex and the time post injury ($F_{3,40}$ =0.1008, p=0.9591). The microglial activation was significantly higher in females compared to males in both sham operated (p=0.0091, p=0.0071 respectively) and brain-injured (p=0.0218, p=0.0025 respectively) cohorts. Furthermore, microglial activation was significantly increased at 1 week post injury in brain-injured mice compared to sham-operated mice in male cohorts (p=0.0383). Overall, this data suggests microglial activation in sham operated mice and brain injured mice was higher in female cohort compared to male cohorts. And the injury induced microglial activation only at 1 week post injury in males.

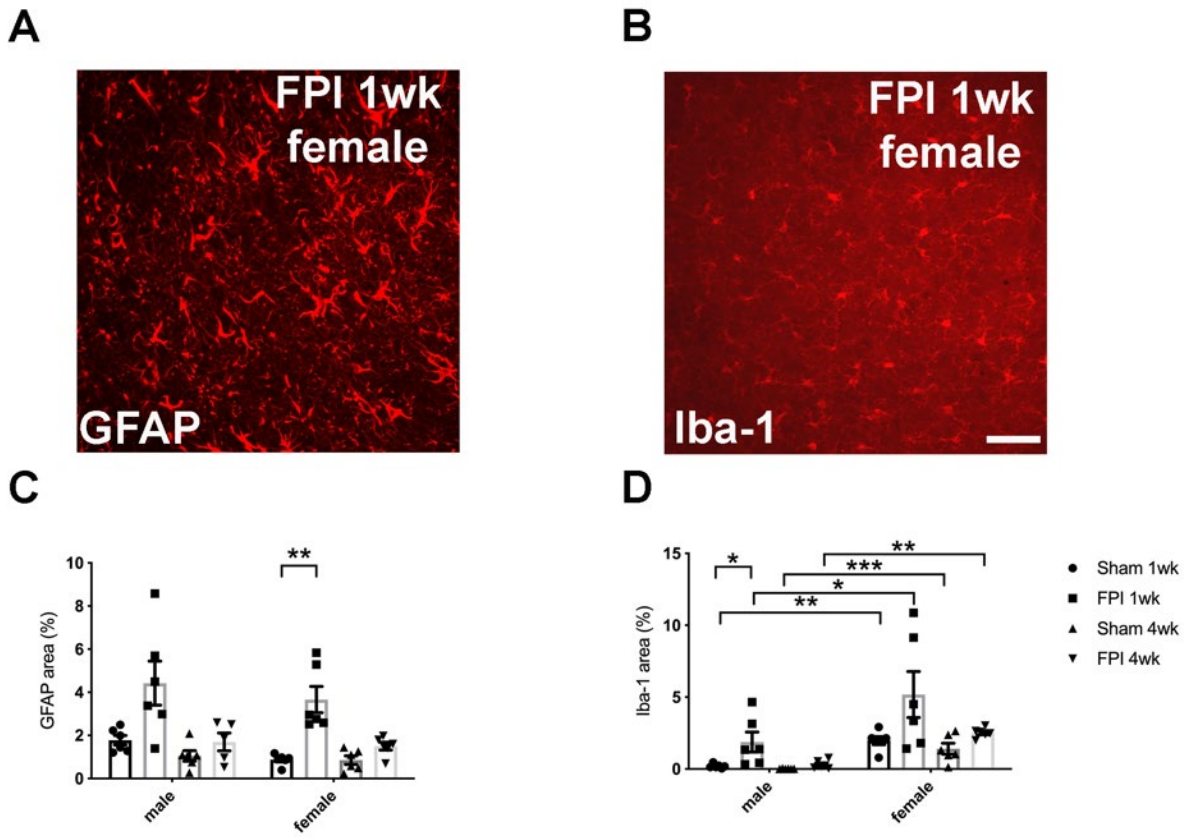

Figure 5.2. Distinctive glial response after mTBI in male and female mice.

*Representative image of mTBI on astrocyte (A) and microglia (B) in the neocortex. In the neocortex, astrocyte activation was increased at 1 week post injury in brain-injured mice compared to sham-operated mice in females. This activation resolved by 4 weeks post injury (C). The microglial activation was significantly higher in female cohorts compared to male cohorts. Furthermore, microglial activation was increased at 1 week post injury in brain-injured mice in comparison to sham-operated mice in males, and this activation resolved by 4 weeks post injury (D). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's test *p<0.05, **p<0.01, ***p<0.001. Scale bar=50um.*

5.3.3. mTBI induced axonal degeneration resolves earlier in female mice but excerbates in male mice

In the current study, axonal degeneration related analysis was investigated in the internal capsule white matter tracts. The amount of axonal degeneration was quantified as a percentage of total area (Fig 5.3 B), an ordinary three-way ANOVA revealed a significant interaction between sex and the time post injury ($F_{3,37}$ =7.360, p=0.0005). Tukey's multiple comparisons tests identified multiple significant differences. The degree of axonal degeneration at 4 weeks post injury in brain injured mice was significantly higher in male brain injured mice compared female brain injured mice (p=0.0002), and was also higher in comparison to male brain injured mice at 1 week post injury (p=0.0157). Furthermore, the axonal degeneration was significantly higher at both 1 week post injury in both males and females brain-injured animals compared to the corresponding sham-operated mice (p=0.0019, p<0.0001 respectively), those was also observed at 4 weeks post injury in both males and females brain-injured mice compared to the corresponding sham-operated mice (p<0.0001, p=0.0139 respectively).

When considering the number of axonal fragments (Fig 5.3 C), an ordinary three-way ANOVA found a significant interaction between sex and the time post injury ($F_{1,37}$ =8.968, p=0.0049). Tukey's multiple comparisons tests identified multiple significant differences. The relative number of axonal fragments in female brain injured mice was significantly decreased from 1 week post injury to 4 weeks post injury (p=0.0050). Furthermore, the relative number of axonal fragments was significantly higher at 1 week post injury in both male and female brain-injured mice compared to the corresponding sham-operated mice (p=0.0481, p<0.0001 respectively), those was also observed at 4 weeks post injury in both males and females brain-injured mice compared to the corresponding sham-operated mice (p=0.0104, p=0.0039 respectively).

Finally, an analysis of the size of axon fragments (Fig 5.3 D) was performed. An ordinary three-way ANOVA found no significant interaction between sex and the time post injury ($F_{1,37}$ =1.934, p=0.1726). The size of axon fragments was not significantly different between males and females in either sham operated or brain injured mice. Furthermore, the size of axonal fragments was significantly increased at 1 week post injury in brain-injured mice compared to sham-operated mice in females (p<0.0001). Additionally, the size of axonal fragments was significantly lower at 4 weeks post injury compared to 1 week post injury in brain-injured mice of females (p<0.0013).

Overall, this data suggests mTBI induced higher axonal degeneration in both male and female mice compared to sham operated mice at both 1 week and 4 weeks post injury. Furthermore,

sex dependent axonal degeneration after mTBI was also observed. While injured induced axonal degeneration in males excerbated overtime, axonal degeneration in females partially resolved.

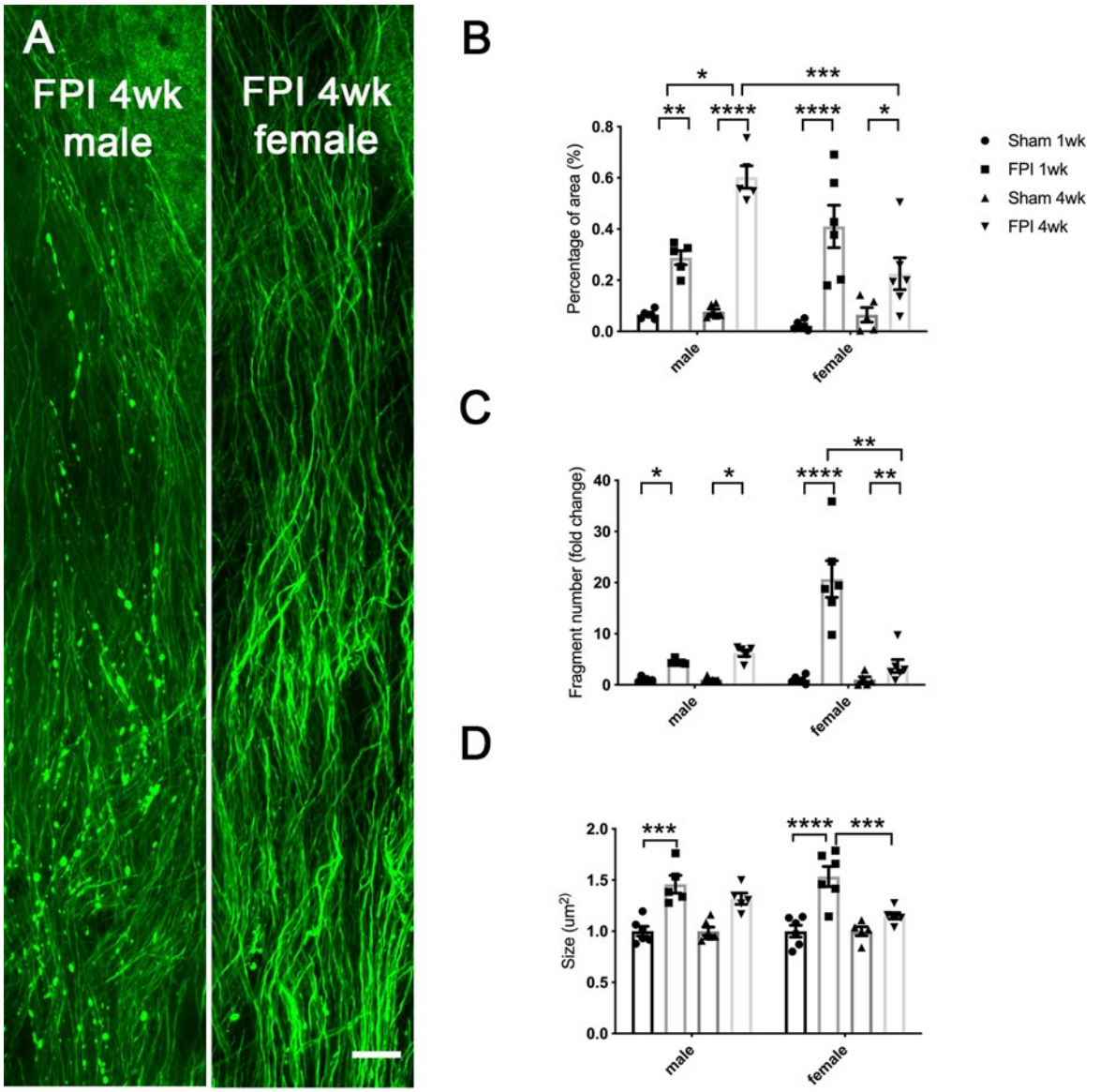

Figure 5.3. Axonal degeneration after injury resolves in females but excerbates in males.

Representative images of YFP expressing axons in the internal capsule in the Thy1-YFPH transgenic mouse brain (A). The percentage of axonal degeneration in the internal capsule was higher at both 1 week and 4 weeks post-injury in both male and female brain injured mice compared to sham operated mice, moreover, the percentage of axonal degeneration at 4 weeks post injury in male brain injured mice, compared to 1 week post injury in male brain injured mice and incomparison to 4 weeks post injury in female brain injured mice (B). The relative number of axon fragments was increased at both 1 week and 4 weeks post-injury in

*both male and female brain injured mice compared to sham operated mice, furthermore, the relative number was significantly higher in female brain injured mice at 1 week post injury compared to 4 weeks post injury (C). The size of axon fragments was increased at 1 week post-injury in both male and female brain injured mice in comparison to sham operated mice. Furthermore, the size of axon fragments was higher in female brain injured mice at 1 week post injury compared to 4 weeks post injury (D). Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's tests *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar=40um.*

5.3.4. Spine density after mTBI was comparable between male and female mice

In the present study, there was no significant difference between total spine density in injured animals compared to shams in either male or female cohorts, at either 1 week or 4 weeks post injury (Fig 5.4 A; $F_{1,41}$ =0.06324, p=0.8027). Similarly for each individual morphological subtype, there were no significant interactions between spines density, sex and time post injury for thin (Fig 5.4 B; $F_{1,42}$ =0.5107, p=0.4788), stubby (Fig 5.4 C; $F_{1,41}$ =0.3119, p=0.5796), or mushroom (Fig 5.4 D; $F_{1,41}$ =0.8388, p=0.3651) spines. Overall, total spine density and specific morphological sub-class spine density did not differ between male and female mice in either sham operated or brain injured cohorts.

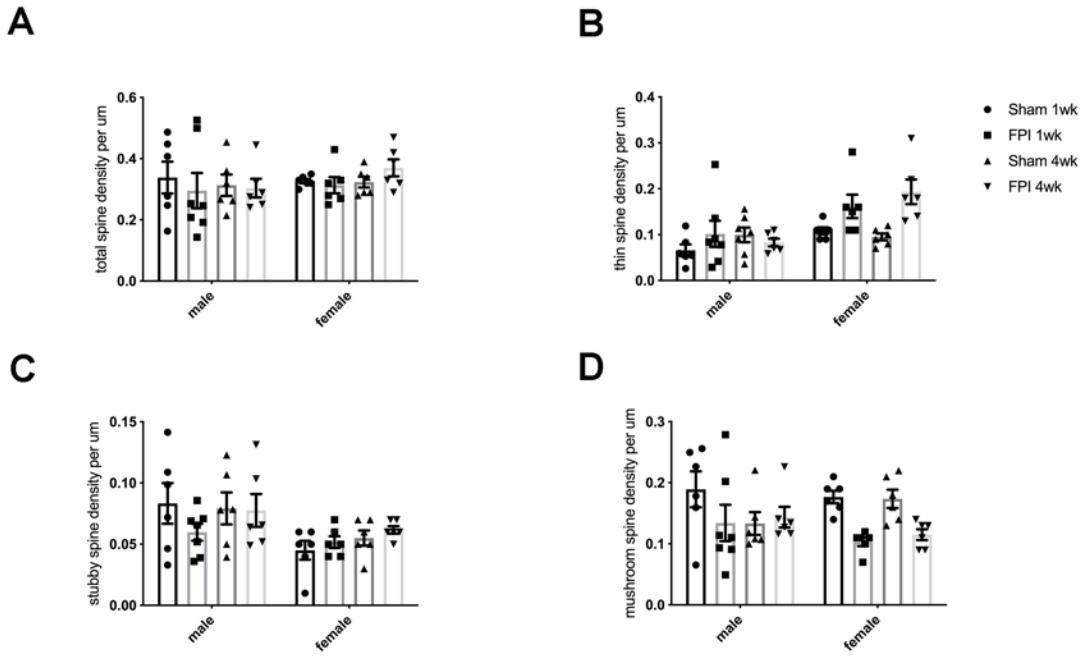

Figure 5.4. Spine density changes after injury are independent of sex.

There was no significant difference between the total spine density (A), or the density of thin (B), stubby (C) and mushroom (D) spines in injured animals compared to shams in either the male and female cohorts, at either 1 week or 4 weeks post injury. Data are presented as mean ± SEM. Three-way ANOVA, followed by Turkey's tests.

5.3.5. Repetitive mTBI resulted in significantly reduced cortical thickness compared to single injury

In the current study, no mortalities were observed in either single injured or repetitive injured animals, but a short time of apnoea (Fig 5.5 A) and a delay in the righting reflex time (Fig 5.2 B) were observed after mTBI. In regard to the duration of apnoea and the righting reflex time (Fig 5.5 A and B), an unpaired t-test found the duration of apnoea and unconsciousness was not significantly different after the second injury compared to the first injury (p=0.0792, p=0.1133 respectively). As to the cortical thickness (Fig 5.5 C), a two-way ANOVA revealed no significant interaction between number of injuries and the time post injury ($F_{1,19}$ =0.06733, p=0.7981). However, the cortical thickness was significantly decreased after repetitive injuries (2 injuries at 48 hrs apart) compared to a single mild injury (p=0.0038). As to the YFP positive cell density (Fig 5.5 D) and the cell soma size (Fig 5.5 E), two-way ANOVA revealed no significantly interaction between number of injuries and the time post injury ($F_{1,19}$ =0.7014, p=0.4127; $F_{1,19}$ =1.441, p=0.2447).

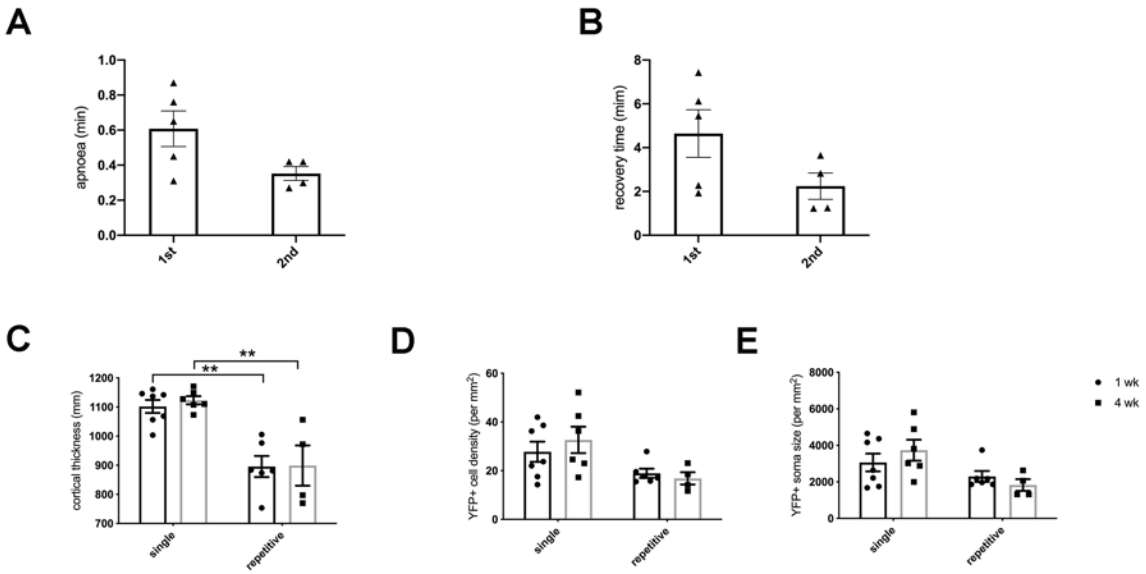

Figure 5.5. Repetitive injury significantly decreased cortical thickness without overt neuronal degeneration compared to a single injury.

*LFPI induced duration of apnoea (A) and righting reflex time (B) did not differ after the second injury compared to the first injury. Repetitive injury (two injuries at 48 hours apart) significantly decreased the thickness of neocortex at both 1 week and 4 weeks post injury compared to a single mild injury (C). An additional injury at 48 hours after the initial injury did not change the YFP positive cell density in the neocortex at either 1 week or 4 weeks post injury in comparison to a single mild injury (D). An additional injury at 48 hours after the initial injury did not alter the YFP positive cell soma size in the neocortex at either 1 week or 4 weeks post injury compared to a single mild injury (E). Data are presented as mean ± SEM. Two-way ANOVA, followed by Tukey's tests **p<0.01.*

5.3.6. Repetitive mTBI resulted significantly increased glial activation in comparison to single injury

The consequence of repetitive injuries (two injuries at 48hrs apart) on GFAP immunoreactivity was investigated within the neocortical region of the injury site (experimental details can be found in Chapter 2). With regard to astrocyte activation (Fig 5.6 C), a two-way ANOVA revealed a significant interaction between the number of injuries and the time post injury ($F_{1,19}$ =7.013, p=0.0159). Repetitive injuries significantly increased astrocytic activation at 1 week post injury in comparison to a single injury (p=0.0060). The astrocyte activation in mice that received repetitive injuries was significantly reduced at 4 week post injury compared to 1 week post injury (p<0.0001), similarly, after a single injury, the astrocytic activation was significantly decreased at 4 weeks post injury compared to 1 weeks post injury (p=0.0457). The consequence of repetitive injuries on Iba1 immunoreactivity was also investigated within the same region of analysis as GFAP (experimental details can be found in Chapter 2). As to the microglial activation (Fig 5.6 D), a two-way ANOVA revealed no interaction between the number of injuries and the time post injury ($F_{1,20}$ =1.151, p=0.2961). However, multiple comparisons showed repetitive injuries significantly increased microglial activation at 4 weeks post injury in comparison to a single mild injury (p=0.0032). Additionally, the microglial activation in mice that received a single injury was significantly decreased at 4 weeks post injury compared to 1 week post injury (p=0.0174). Overall, compared to a single injury, repetitive injuries significantly increased astrocytic activation at 1 week post injury and resolved at 4 weeks post injury, and the injuries increased microglial activation at 4 weeks post injury.

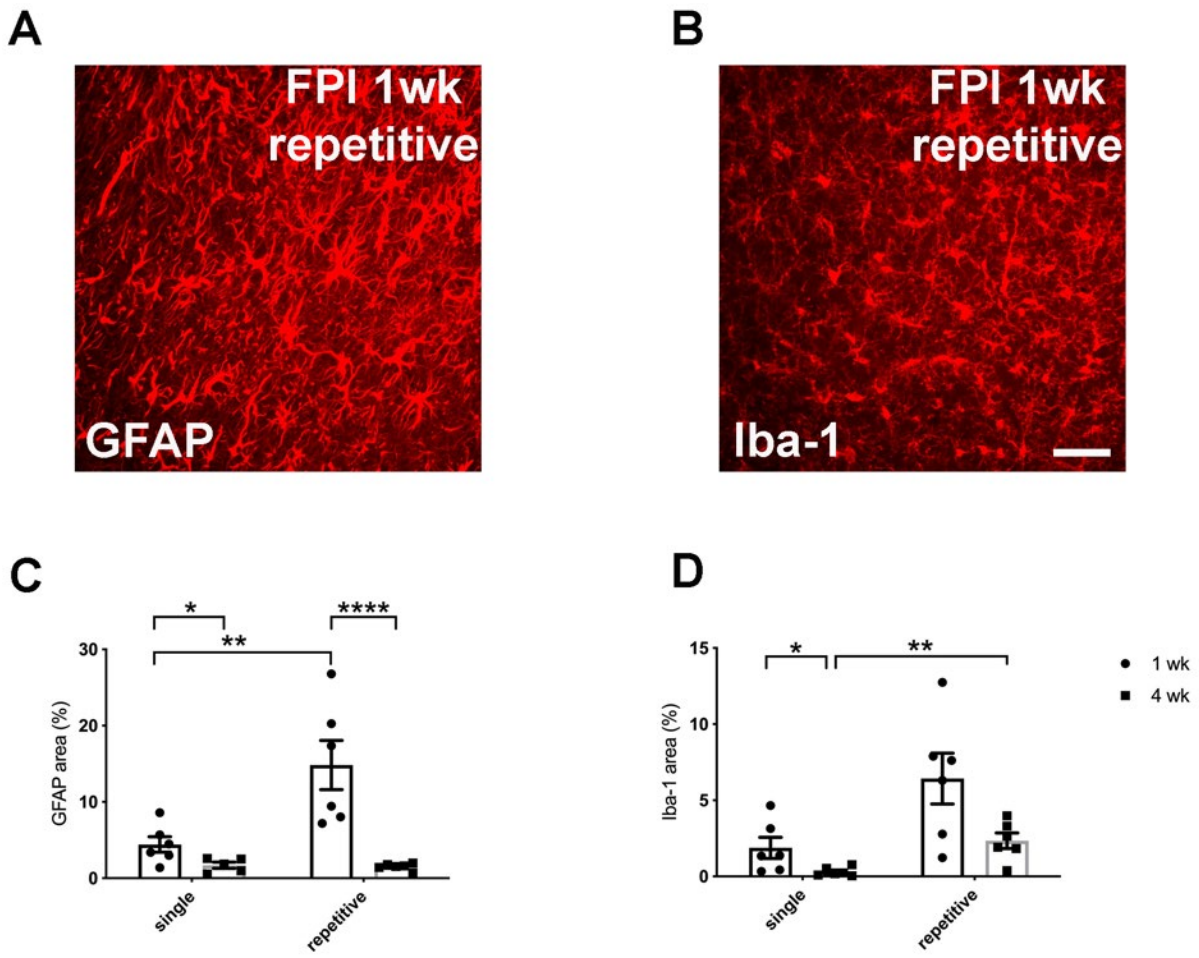

Figure 5.6. Repetitive injury significantly increased astrocytic and microglial activation compared to a single injury.

*Representative image of repetitive injury (two injuries at 48 hours apart) on astrocyte (A) and microglia (B) in the neocortex. Repetitive injuries significantly exacerbated astrocytic activation in the injured neocortex at 1 week post injury in comparison to a single mild injury (C). An additional injury at 48 hours after the initial injury significantly exacerbated microglial activation at 4 weeks post injury in the injured neocortex in comparison to a single mild injury (D). Data are presented as mean ± SEM. Two-way ANOVA, followed by Tukey's tests *p<0.05, **p<0.01, ****p<0.0001. Scale bar=50um.*

5.3.7. Repetitive mTBI resulted in significantly exacerbated axonal degeneration in comparison to a single injury

In the present study, analysis of the percentage area of axonal degeneration (Fig 5.7 B) by two-way ANOVA revealed no significant interaction between the number of injuries and the time post injury ($F_{1,15}$ =0.7062, p=0.4139). The axonal degeneration was significantly increased after repetitive injuries (2 injuries at 48 hrs apart) in comparison to a single mild injury at 1 week post injury (p=0.0079). In regard to the relative number of axon fragments (Fig 5.7 C), a two-way ANOVA revealed no significant interaction between number of injuries and the time post injury ($F_{1,15}$ =0.6817, p=0.4219). Finally, in regard to the size of axon fragments (Fig 5.7 D), a two-way ANOVA revealed no significant interaction between number of injuries and the time post injury ($F_{1,15}$ =3.200, p=0.0938). However, the axonal fragment size was significantly increased after repetitive injuries compared to a single mild injury at both 1 week and 4 weeks post injury (p<0.0001, p=0.0115 respectively).

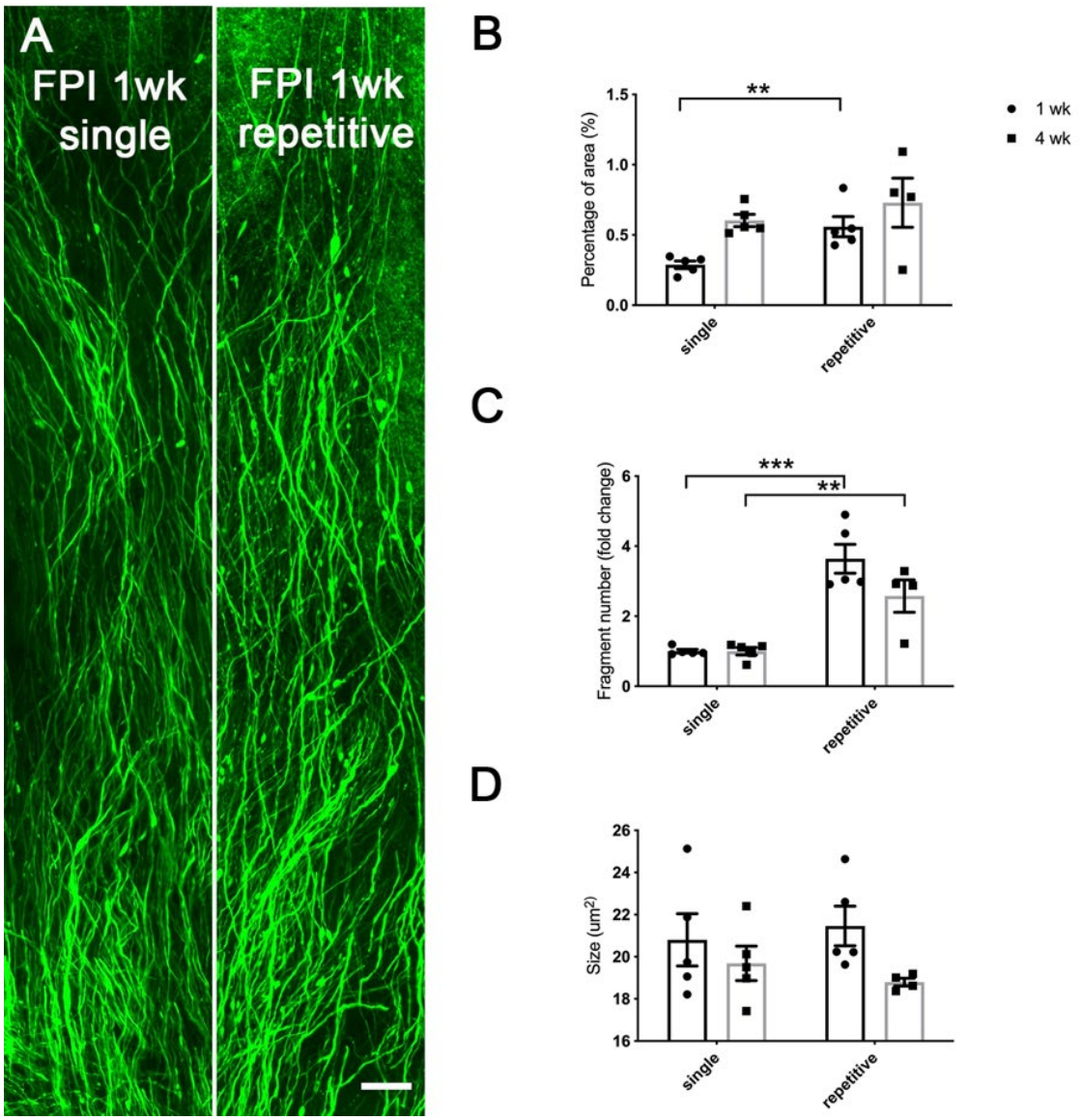

Figure 5.7. Repetitive injuries significantly exacerbated axonal degeneration compared to a single injury.

*Representative images of YFP expressing axons in the internal capsule of the Thy1-YFPH transgenic mouse brain (A). Repetitive injury (two injuries at 48 hours apart) significantly increased the percentage area of axonal degeneration in the internal capsule at 1 week post injury but not at 4 weeks post injury in comparison to a single mild injury (B). A second injury at 48 hours after the initial injury significantly increased the number of axonal fragments at both 1 week and 4 weeks post injury in comparison to a single mild injury (C). A second injury at 48 hours after the initial injury did not change the size of axonal fragments in the internal capsule at either 1 week or 4 weeks post injury in comparison to a single mild injury (D). Data are presented as mean ± SEM. Unpaired t-test, **p<0.01, ***p<0.001. Scale bar=40um.*

5.3.8. Repetitive mTBI did not change spine density compared to a single injury

In the present study, there was no significant difference between total spine density in repetitive injured (2 injuries at 48 hrs apart) animals compared to single injured animals, at either 1 week or 4 weeks post injury (Fig 5.8 A; $F_{1,19}$ =0.1944, p=0.6643). Similarly for each individual morphological spine subtypes, there was no significant interaction between spine density, injury numbers and the time post injury for thin (Fig 5.8 B; $F_{1,19}$ =0.008780, p=0.9263), stubby (Fig 5.8 C; $F_{1,19}$ =0.1951, p=0.6637), or mushroom (Fig 5.8 D; $F_{1,19}$ =0.5200, p=0.4796) spines. Overall, repetitive brain injury did not change the total spine density or sub-class specific spine density compared to a single mild injury.

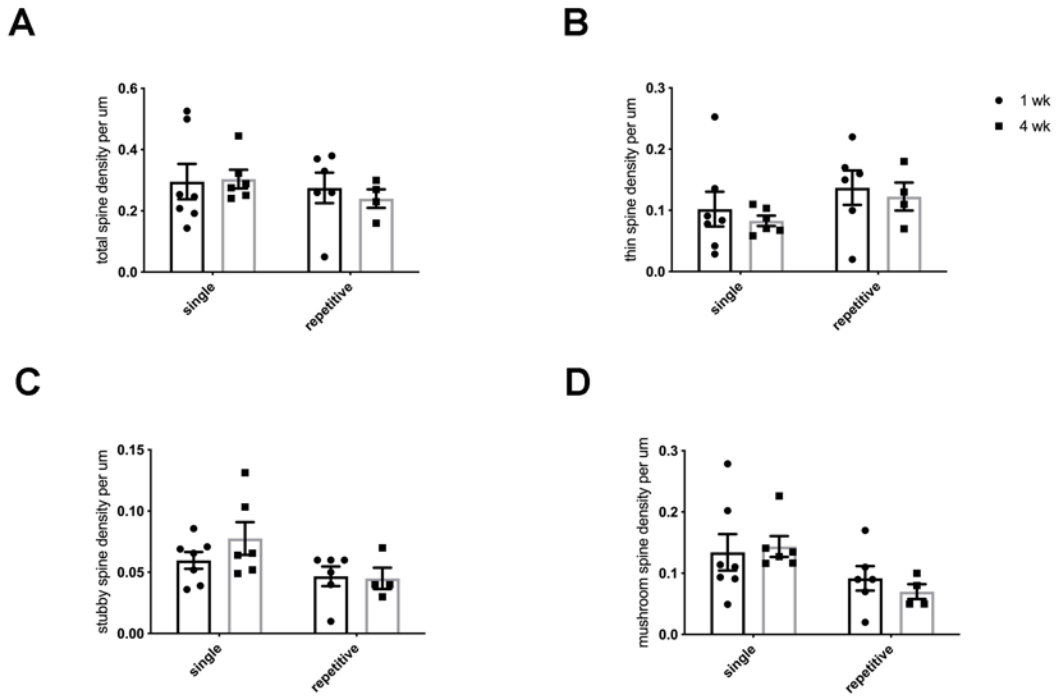

Figure 5.8. Repetitive injury did not affect the density of total spines or the individual sub-classes of spines compared to a single injury.

No significant changes between the total spine density (A), as well as the density of thin (B), stubby (C) and mushroom (D) spines after repetitive injuries (two injuries at 48 hours apart) compared to a single mild injury at either 1 week or 4 weeks post injury. Data are presented as mean ± SEM. Two-way ANOVA.

5.4. DISCUSSION

Males are reported as having a higher possibility of sustaining TBI than females during their lifespan (Rickels et al., 2010), however, females have been reported with a 1.4 times higher incidence of concussion than males in some sports activities (Covassin et al., 2016). Outcomes after brain injuries between sexes are complicated and inconsistent, with most clinical studies showing females having poorer outcomes following TBI, including mild severity injuries, while a number of experimental studies suggest females may have reduced negative outcomes (Wagner et al., 2004, Bruce-Keller et al., 2007, Dick, 2009, Preiss-Farzanegan et al., 2009, Bazarian et al., 2010, Cantu et al., 2010, Frommer et al., 2011, Laker, 2011, Covassin and Bay, 2012). Therefore, investigating sub-cellular reactions following mild injury in animals may help us understand how sex can influence outcomes following mild TBI.

Repetitive mTBI (rmTBI) has been associated with neurophysiological changes, cognitive impairments, and the development of neuronal degenerative diseases in later life (including AD and CTE) (Luo et al., 2014, Nordström et al., 2014, Vynorius et al., 2016, Multani et al., 2016, Montenigro et al., 2017, Merritt et al., 2018). Compelling studies have tried to replicate the biomechanical, neurological and pathological changes of rmTBI patients, with inconsistent number of injuries and intervals between injuries used. Thus, additional studies on repetitive mild injured are also needed. The final chapter of this thesis, presents two further investigations of potential risk factors for mTBI, sex and a subsequent second injury.

In the present study, using a mild LFPI model in both males and females, we observed a thinner cortex without overt neuron loss in females compared to males. Additionally, the glia showed distinctive activation pattern after mild injury between male and female mice, however, axonal degeneration after mTBI is initiated at short-term in both sex, and later on, excerbated in males but partially resolved in females. Furthermore, we found repetitive injury (two injuries at 48 hrs apart) significanlty increased astrocytic activation at 1 week after injury and microglial activation at 4 weeks post injury. Additionally, repetitive injury also reduced cortical thickness and exacerbated axonal degeneration at 4 weeks post injury compared to a single mild injury. Thus it appears that repetitive injury, 48 hours apart, exacerbates cellular and subcellular pathology.

The lateral fluid percussion injury model has been widely used to mimic clinical mTBI pathologies (Spain et al., 2010, Alder et al., 2011, Shultz et al., 2011, Wright et al., 2016, Powell et al., 2018). In both clinical and preclinical studies, a TBI is considered mild if it occurs in the absence of overt cortical loss and cavitation (Iverson et al., 2010, Gao and Chen, 2011,

Meconi et al., 2018). Additionally, it has also been suggested that the mortality rate after experimental mild injury should be between 0 and 5%, with a righting reflex within 2 and 4 minutes and a loss of consciousness of less than 2 minutes (Morehead et al., 1994, Alder et al., 2011, Teng and Molina, 2014, Katz et al., 2015). In the current study, mortality, loss of consciousness and righting reflex results validated that the injury in our study was mild in severity. It has been reported that the duration of apnoea and righting reflex time was not significantly different between male rats and female rats following moderate TBI (Avcu et al., 2019, Newell et al., 2019, Saber et al., 2019). Similarly, in the current study, the duration of apnoea and righting reflex time was comparable between female and male brain-injured mice, thus providing further evidence for support of the use of the lateral fluid percussion injury model in both sexes of mice in future studies.

In order to investigate the discrepancies in the literature between clinical and pre-clinical studies in regard to the effect of sex and also the effect of an additional injury on mTBI outcomes, we firstly investigated the neuronal population. Absence of overt neuronal loss after mTBI has been widely observed in male brain injured mice (Iverson et al., 2010, Gao and Chen, 2011, Meconi et al., 2018). Similar to a recent previous study (Velosky et al., 2017), we found female sham mice had reduced cortical thickness compared to male sham mice. A study by Wright and colleagues reported reduced thickness of the prefrontal cortex at 7 days after multiple mTBIs in female rats (Wright et al., 2018). Additionally, it has also been reported that higher hemispheric volume loss was detected at 21 days but not at 1 day after moderate to severe TBI in both male and female mice (Newell et al., 2019). In the present study, we found that mild injury did not change cortical thickness at 1 week post injury, however it significantly decreased cortical thickness at 4 weeks post-injury compared to shams in female mice, which was not observed in male mice. Additionally, the YFP positive cell density and soma size did not change after injury in either female or male mice. Thus, it may suggest mTBI in female mice may result in later onset decrease in the cortical thickness but without overt changes in pyramidal neurons.

With regard to repetitive mTBI (rmTBI), a number of previous studies have reported no overt structural abnormalities or neuronal loss after rmTBI (Huang et al., 2013, Weil et al., 2014, Winston et al., 2016, Xu et al., 2016, Effgen and Morrison, 2017, Robinson et al., 2017, Gold et al., 2018, Meconi et al., 2018), whereas others report hemorrhagic lesions and degenerating cells at short term as well as longer term timer points after rmTBI (Aungst et al., 2014, Bolton and Saatman, 2014). In the present study, we found repetitive mild injuries (an additional injury at 48 hours intervals) resulted in a significantly reduced cortical thickness relative to a single mild injury at both 1 week and 4 weeks post injury. This suggests that a second injury at 48

hours after the initical injury caused severe damage to the neocortex. One study reported rmTBI (5 injuries at 48 hours intervals) caused comparable neurodegeneration to those of a single mild injury at 9 days post injury (Bolton and Saatman, 2014). Similarly, we observed that the repetitive injury did not change the YPF positive cell density and cell soma size in the cortex compared to a single mild injury, suggesting no overt pyramidal neuron loss in the cortex after repetitive injury. Overall, our studies further supported that a second injury at 48 hours intervals significantly reduced cortical thickness without overt changes in pyramidal neuronal populations compared to a single mild injury.

Next, we investigated the effect of sex and an additional injury on the glial reaction after mTBI. Increased levels of GFAP and Iba-1 are an indication of reactive astrogliosis and microgliosis respectively (Liedtke et al., 1996, Ito et al., 1998). According to the literature of mTBI, higher astrocytic and microglial activation have been observed at both short term and long term time points after brain injury (Shultz et al., 2013, Huang et al., 2014, Goodrich et al., 2016, Rodriguez-Grande et al., 2018). Glial activation plays an important role in both promoting and preventing the recovery from brain injury (Womble and Moises, 1993, Hamlin et al., 2001, Bayir et al., 2007, Wu et al., 2009, Pun et al., 2010, Burda et al., 2016, Loane and Kumar, 2016). It has been reported that astrocyte activation is higher in female mice at 1 day post moderate TBI compared to shams and male brain injured mice, but this difference was not observed at 7 days post-injury (Jullienne et al., 2018). However, other investigations have reported that astrocytes activation is higher in males compared to females at 7 days after injury, but not at 30 days, in the cortex, and that this sex related difference was not observed in the dentate gyrus (Villapol et al., 2017). In the current study, we found there was higher astrocytic activation in the somatosensory cortex at 1 week post-injury in brain injured mice compared to sham operated mice in female cohorts. Moreover, this injury related astrocytic activation resolved at 4 weeks post-injury. Our results suggest male and female mice showed different patterns of astrocytic activation after mild TBI. In an investigation using the controlled cortical impact model of injury, it has been reported that microglial activation is higher in both male and female mice at 7 days but not at 1 day post moderate TBI compared to shams, and is not sex dependent (Jullienne et al., 2018). Moreover, it is also reported that a moderate-severe TBI increased microglial activation in the cortex among both male and female mice at 1 day post injury but not at 21 days post injury and was not sex dependent (Newell et al., 2019). Furthermore, another study found microglial activation after a moderate to severe TBI in the cortex was not different between male and female brain-injured mice at either 7 days post injury or 30 days post injury (Villapol et al., 2017). In the current study, we found there was higher microglial activation in the injured cortex at 1 week post-injury compared to sham mice in male mice. Moreover, the microglial activation in sham operated mice and brain injured

mice was higher in female in comparison to male cohorts. Cumulatively, this data suggests that male and female mice showed distinct patterns of astrocytic and microglial activation after mild TBI.

In terms of rmTBI, widespread astrogliosis has been observed at both short and long term post-injury time points, and at multiple sites including the cortex, corpus callosum and hippocampus (Luo et al., 2014, Yang et al., 2015, Xu et al., 2016, Chen et al., 2017, Effgen and Morrison, 2017, Robinson et al., 2017, Cheng et al., 2019). Furthermore, widespread Iba-1 positive cells have been reported in multiple sites both at short term and long term after rmTBI in the cortex, optic tract and the external capsule (Bolton and Saatman, 2014, Fidan et al., 2016, Winston et al., 2016, Xu et al., 2016, Chen et al., 2017, Effgen and Morrison, 2017, Robinson et al., 2017, Cheng et al., 2019). Similarly, in the current study, we observed that repetitive mild injuries (an additional injury at 48hrs post initial injury) significantly increased astrocytic at 1 week post injury and microglial activation at 4 weeks post injury in the injured cortex compared to a single mild injury. Furthermore, Bolton and Saatman reported that rmTBI (5 injuries at 48hrs intervals) caused comparable astrocytic activation to those of a single mild injury at 9 days post injury (Bolton and Saatman, 2014). In the current study, we observed both that the astrocytic activation after rmTBI was similar to those of a single mild injury at 4 weeks post injury.

Numerous studies have reported dendritic changes, including dendrite loss and degeneration and changes to spine turn over, after mTBI (Cahill et al., 2016, Gao and Chen, 2011, Sword et al., 2013, Chen et al., 2018, Zhao et al., 2018). In one study, it was reported that there was no difference in dendritic spine density between sexes at 20 days after mTBI in juvenile (P30) rats (Mychasiuk et al., 2015b). However, others found the spine density was not different between sexes in juvenile (P48) sham operated mice, but the total spines density in the mediate medial prefrontal cortex as higher in brain injured mice at 17 days after injury (using closed head weight drop model) in females than males (Hehar et al., 2015). In present study, we found there was no difference in total spine density and sub-class specific of spine density between male and females and between injury and shams. The discrepancy may due to the method used - Hehar and colleague used a modified close head weight drop injury model and applied Golgi-Cox straining for quantification. With regard to rmTBI, studies report repetitive injuries exacerbated dendritic degeneration and loss, as well as spine density reduction (Ojo et al., 2015, Effgen and Morrison, 2017, Algamal et al., 2019). In the current study, we did not observe any significant changes in total spine density and subclass spine densities after an additional injury at 48 hours compared to a single mild injury. The discrepancy may due to the

method of quantitation used, as Golgi-Cox Staining and electron microscopy was used in other studies (Ojo et al., 2015, Algamal et al., 2019).

Lastly, we investigated the axonal responses after mTBI, relative to sex and the proposed risk factor of an additional injury. Diffuse axonal injury is a pathological hallmark of mTBI. It has been suggested that axon impairments with transport disruption, organelle accumulation, axonal swelling, bulb formation and degeneration following mTBI are associated with the behavioural and cognitive deficits after brain injury (Carmona et al., 2010, Greer et al., 2013, Mierzwa et al., 2015, Henninger et al., 2016, Chen et al., 2018, Chuckowree et al., 2018). However, studies of axonal pathology after injury have been mainly conducted in male animals while female animals are less well investigated. In an *in vitro* model of traumatic axonal injury (TAI) in neurons derived from both rat primary cortical neurons and human induced Pluripotent Stem Cells (iPSC), it was reported that the stretch injury induced significantly more swellings and increased loss of calcium signaling function in axons derived from female rat primary cortical neurons and female differentiated iPSC compared to males at 24 hours post-injury (Dollé et al., 2018). Moreover, it is reported by Newell and colleagues, a moderate to severe TBI in juvenile mice (21±3 days) resulted in a significant increase in the percentage area of βAPP in the corpus callosum and external capsule at both 1 day and 21 days post injury but this result was not different between male and female brain-injured mice (Newell et al., 2019). In the current study, we observed that mTBI significantly increased axonal degeneration at both 1 week and 4 weeks post injury in both male and female cohorts. However, sex dependent axonal degeneration was also indicted. Increased axonal degeneration was observed at 4 weeks post injury in males compared to females brain injured mice. Moreover higher axonal degeneration in male brain injured mice at 4 weeks post injury was found compared to 1 week post injury, while less fragment number and smaller fragment size were observed in female brain injured mice at 4 weeks post injury in comparison to 1 week post injury. Overall, our findings suggest that axonal degeneration after mTBI initates at 1 week post injury in both male and female cohorts, while at later timepoint, resolved earlier in female cohorts but excerbated in male cohorts.

In regard to rmTBI, axonal pathology has also been widely reported, which may partially contribute to injury-related behavioural and cognitive deficits (Fujita et al., 2012, Hylin et al., 2013, Bennett and Brody, 2014, Bolton and Saatman, 2014, Luo et al., 2014, Mouzon et al., 2014, Fidan et al., 2016, Chen et al., 2017, Cheng et al., 2019, Cross et al., 2019). It has been reported that the axonal damage detected by APP immunolabeling was more predominant with shorter intervals between injuries (3 hours and 24 hours intervals) (Fujita et al., 2012,

Bolton and Saatman, 2014). Additionally, it has also been reported that rmTBI induced increased phosphorylation of tau, and axonal argyrophilia in the external capsule and corpus callosum compared at 1 week post injury (Fidan et al., 2016, Tan et al., 2016). In the current study, we observed that axonal degeneration in the internal capsule is increased at 1 week but not at 4 weeks post repetitive injury (two injuries at 48hrs apart) compared to a single injury. Additionally, axonal fragment number was higher at both 1 week and 4 weeks post repetitive injury in comparison to a single mild injury. Therefore, it indicates that the second injury significantly exacerbated the axonal degeneration in the internal capsule at both 1 week and 4 weeks post injury. Our findings contrast with those of Bolton and colleagues (Bolton and Saatman, 2014). In their study, they applied 5 mild closed head injuries at 48 hours apart, and they found the pathological outcomes at 9 days after the initial injury after 5 injuries was comparable to a single mild injury (Bolton and Saatman, 2014). The discrepancy between our findings and Bolton and colleagues' findings is possibly due to the model used. In the current study we used an open head mild injury model, whereas Bolton and colleagues applied a closed head mild injury model. This may suggest that repetitive injury (two injuries at 48 hours intervals) initiated axonal pathological changes earlier and persisted longer in the internal capsule white matter tracts. However, future studies including repetitive sham operated mice are needed in order to verify this.

To conclude, in the present study, we found that glial responses to trauma were sex dependent. Additionally, axonal degeneration in the cortex triggered by mild traumatic brain injury was sex dependent with the degeneration initiated at short-term in both male and female cohorts, resolved quicker in females but excerbated in males. Additionally, we provide further evidence supporting previous findings that repetitive injury (two injuries at 48hrs apart) significantly exacerbates cellular and sub-cellular pathologies, with increased astrocytic activation short-term after injury and microglial activation long-term after injury, as well as decreased cortical thickness and increased axonal degeneration at both 1 week post injury in comparison to a single brain injury. Therefore, more attention and monitoring are needed for mTBI patients at risk of secondary injuries.

6. DISCUSSION

mTBI, accounting for more than 80% of TBI cases (Luo et al., 2017), is a serious public health issue (Gardner and Yaffe, 2015). According to the Centers for Disease Control and Prevention (CDC), children aged under 4 years, adolescents aged between 15 and 19 years, together with older aged people over 75 years have high incidence rates of TBI (Laskowitz and Grant, 2016). With a growing ageing population, the number of aged TBI cases is increasing. Previous studies have reported that being older in age at the time of injury is associated with higher mortality and morbidity, as well as increased behavioural and cognitive impairments. However, in the context of mTBI, the influence of age at the time of injury on the outcomes following injury is less well investigated and the underlying cellular changes are less well studied. Whilst the therapeutic potential of epothilone D in mTBI has been investigated in both *in vitro* and *in vivo* studies, whether there exists an age dependent effect of epothilone D has not been well studied. Previous studies have reported that there are differences related to sex not only in the incidence but also in the recovery processes that occur after brain injury (Hillbom and Holm, 1986, Hirtz et al., 2007). However, with regard to mTBI, the influence of sex on the cellular reaction is less well studied. Furthermore, various parameters, such as the number of injuries and the interval between injuries, have been investigated in models of repetitive mTBI (Cheng et al., 2014a, Selwyn et al., 2016, Multani et al., 2016, Ferguson et al., 2017a, Merritt et al., 2018), however, the reports regarding the resulting cellular and subcellular changes are still inconclusive and inconsistent.

The outcomes and recovery processes following mTBI are heterogeneous, this is partially due to the interplay of various risk factors. Thus, this thesis using lateral fluid percussion injury, focused on the neuronal and glial reactions after injury to provide insights into the cellular and subcellular responses to mTBI in the brain. The hypothesis of this thesis was that cellular and subcellular responses to mTBI, of both neuronal and glial populations, vary greatly depending on a variety risk factors, including age at injury, post-injury time interval, sex, repetition of injury and duration between injuries. In order to address the hypothesis, this thesis investigated four aims. In Aim 1, to determine the effect of age on the cellular reactions after mTBI in a mouse model, a mild lateral fluid percussion injury was performed in young (1.5 months), adult (3 months) and aged (12 months) mice, with cellular and subcellular responses compared between brain injured mice and sham operated mice, as well as between different aged mice (Chapter 3). Then to extend upon the findings of Aim 1, and determine the age dependent therapeutic potential of the microtubule stabilizing agent epothilone D following mTBI in young (1.5 months) and adult (3 months) mice, as described in Aim 2 of this thesis, the effect of epothilone D or vehicle treatment was measured by neuronal and glial reactions following

mTBI in both young and adult cohorts (Chapter 4). Finally, two studies investigated how the potential risk factors, sex (Aim 3) and an additional injury (Aim 4), influence the cellular and subcellular reactions after mild lateral fluid percussion in a mouse model (Chapter 5).

We found that the risk factors, age at injury, sex and additional injury, consistently did not differentially affect the duration of apnoea or the period of unconsciousness after mTBI. Additionally, these factors did not differentially influence the pyramidal neuronal population, with regard to pyramidal cell number or cell soma size, in the neocortex at either 1 week or 4 weeks post injury. Furthermore, these factors did not differentially affect the density of total dendritic spines or specific sub-classes of spines at either 1 week or 4 weeks post injury.

It was, however, found that these three risk factors differentially affected the axonal response after mTBI. With regard to the age at injury, injury induced axonal degeneration in the internal capsule was found to be age dependent. Whilst axonal degeneration was observed at 1 week post injury but resolved at 4 weeks post injury in young brain injured mice, it was observed at both 1 week and 4 weeks post injury in both adult and aged brain injured cohorts. Additionally, axonal degeneration at 4 weeks post injury in the internal capsule was higher in both adult and aged brain injured mice compared to young brain injured mice (summarized in Fig 6.1). Furthermore, the effect of epothilone D treatment on axonal degeneration after injury was age dependent, with no significant positive or negative effect in the young brain injured mice, but significantly exacerbated axonal degeneration in adult brain injured mice compared to vehicle treated animals (summarized in Fig 6.2). As to the influence of sex, we found that mTBI induced axonal degeneration initiated at short term both in male and female cohort, and was partially resolved in females while was excerbated in males at long term after injury (summarized in Fig 6.3). Finally, with regard to the influence of an additional injury, we found that repetitive injury (two injuries at 48hrs apart) significantly exacerbated axonal degeneration in the internal capsule in comparison to a single mild injury (summarized in Fig 6.4).

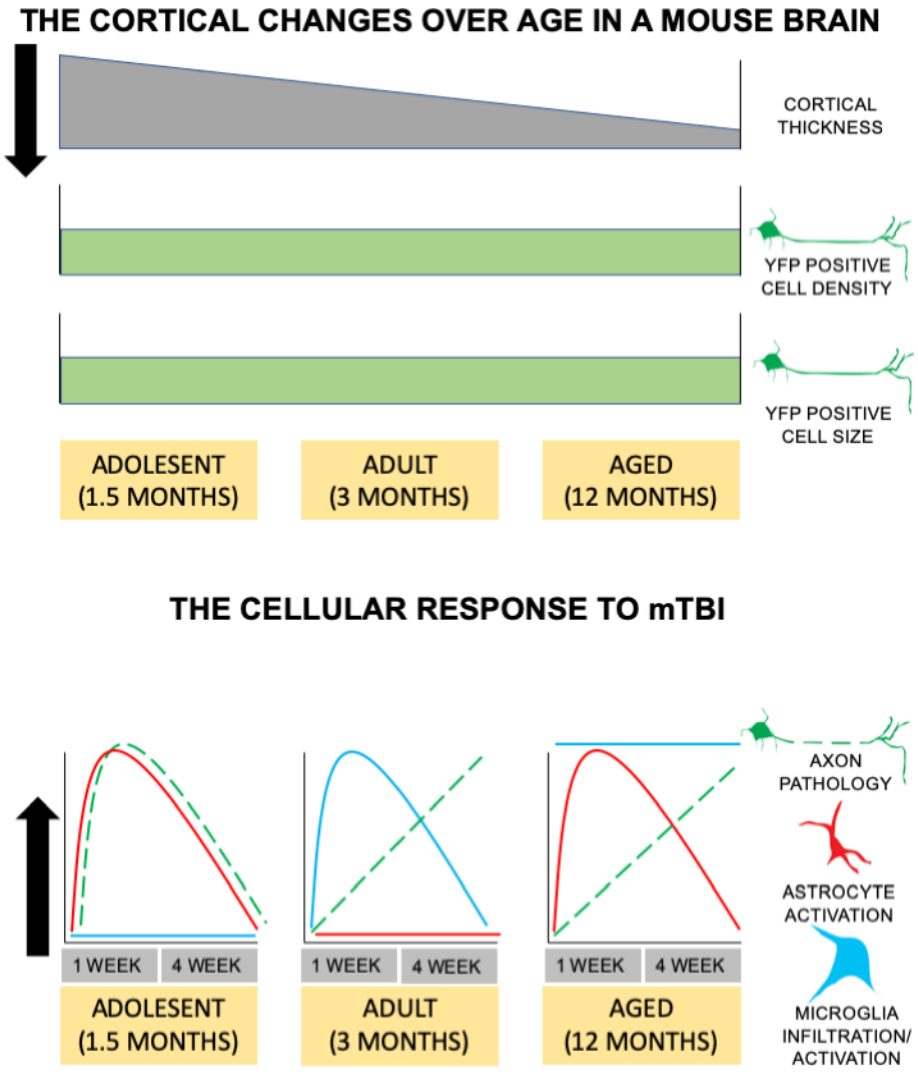

Figure 6.1 Graphical abstract for Aim 1.

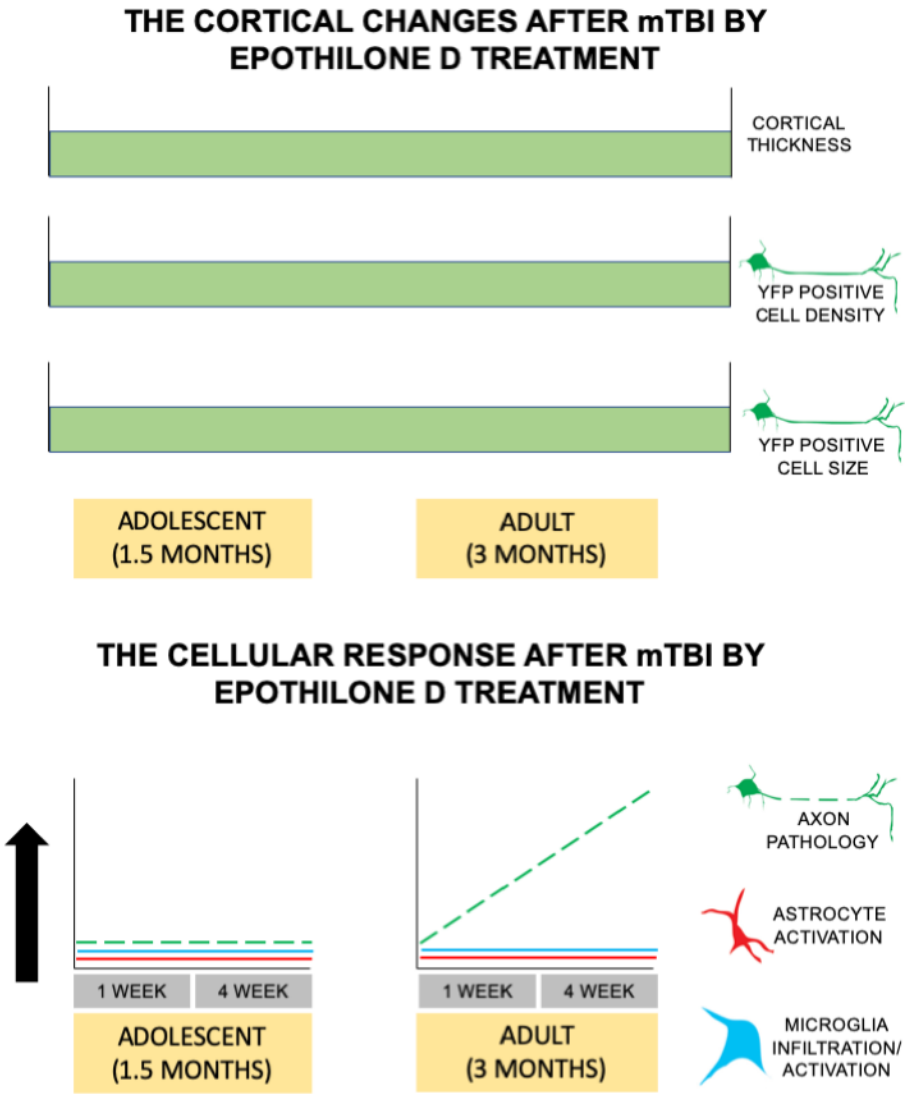

Figure 6.2 Graphical abstract for Aim 2.

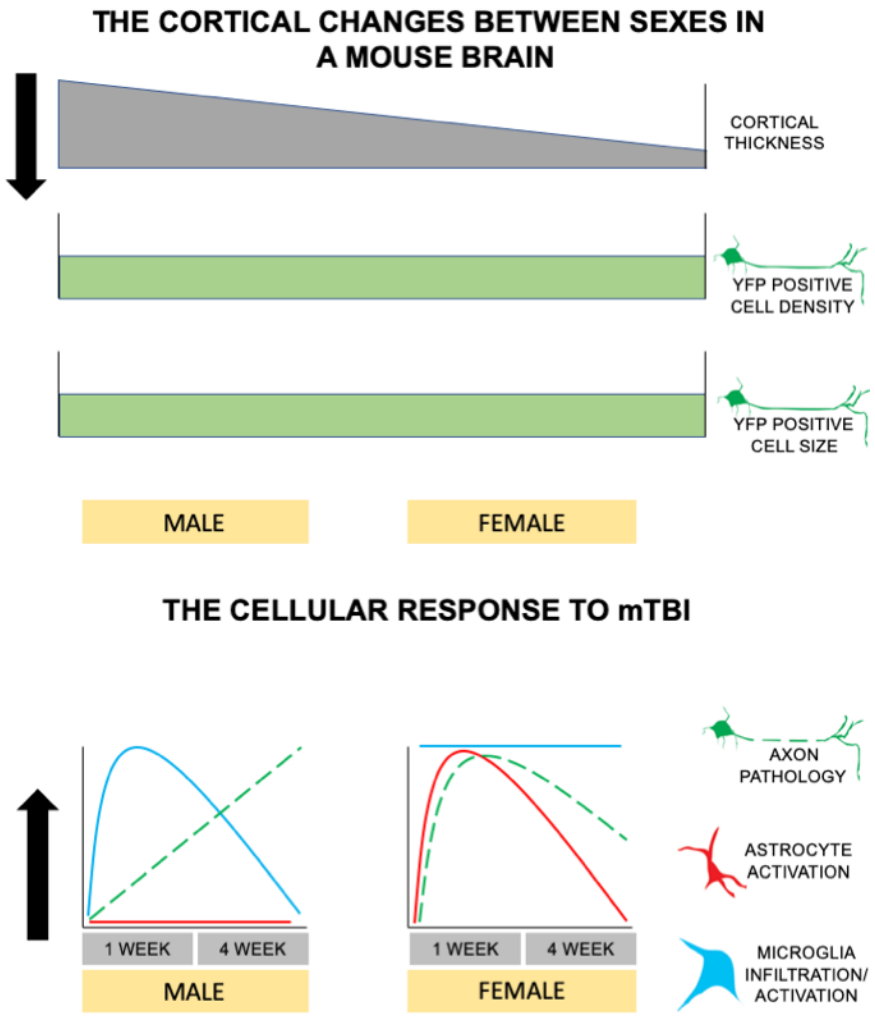

Figure 6.3 Graphical abstract for Aim 3.

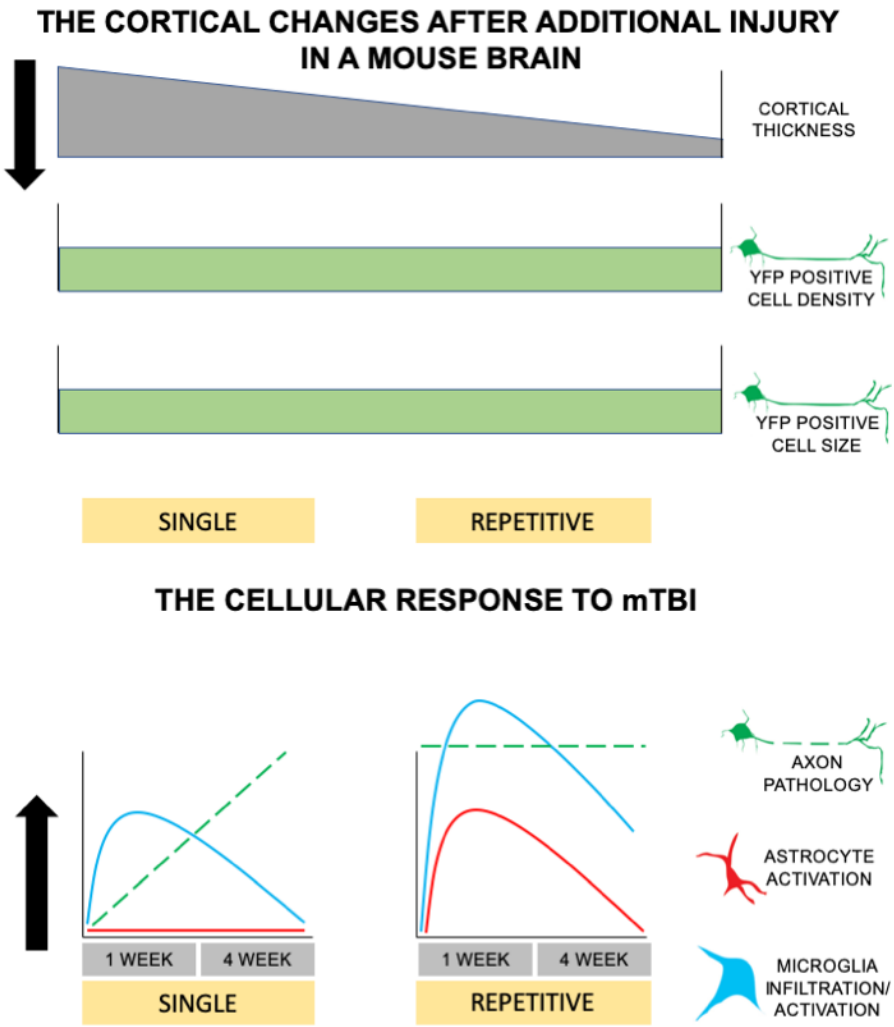

Figure 6.4 Graphical abstract for Aim 4.

Age at injury, sex and and an additional injury were also found to differentially influence the glial response to mTBI. With regard to the influence of the age at injury, glial activation was age dependent, with higher astrocyte activation in young brain injured mice but not in either adult or aged brain injured mice. Additionally, mTBI increased microglial activation in adult brain injured mice but not in young or aged brain injured mice compared to their sham operated counterparts, the microglial activation in both sham operated and brain injured was higher in aged mice compared to younger mice (summarized in Fig 6.1). With regard to the influence of sex, we found that mTBI induced astrocyte activation was dependent of sex, with injury induced higher astrocyte activation at 1 week post injury in female but not in male cohort. Additionally, microglial activation was also found to be dependent of sex. Microglial activation was higher in male but not in female brain injured mice compared to sham operated mice at 1 week post injury, and the microglial activation in both sham operated and brain injured was higher in female cohorts compared to male cohorts (summarized in Fig 6.3). Finally, investigations of the influence of an additional injury found that repetitive injury significantly increased astrocytic activation at 1 week post injury and microglial activation at 4 weeks post injury in comparison to a single mild injury (summarized in Fig 6.4).
We did not find any differential influence of the age at injury on the cortical thickness. However, with regard to the influence of sex, we found that the cortical thickness in female brains was thinner compared to male mice brains, and the injury resulted in a decrease in cortical thickness at 4 weeks post injury in female, but not male, mice (summarized in Fig 6.3). Finally, we found that repetitive injury significantly decreased the cortical thickness compared to a single mild injury in males (summarized in Fig 6.4).

Several notes need to be mentioned in the current study. First, injury related results in chapter 3 were not replicable in chapter 4. A possible reason for the inconsistences between some results presented in Chapter 3 compared to those in Chapter 4 could be the effect of DMSO, which was used as the vehicle and vehicle control in the Epothilone investigations. Windrum and colleagues have found that reducing the concentration of DMSO prior to treatment could significantly alleviate adverse reactions to cells (Windrum et al., 2005), however, further studies exploring the effect of DMSO on neurons and astrocytes have reported that there was cell toxicity even at low DMSO concentrations, including neurotoxic effects (Wingerchuk and Carter, 2014), neurophysiological alterations in pyramidal neurons (Tamagnini et al., 2014), and activation of astrocytes (Zhang et al., 2017a). Therefore, in the current study, the somewhat inconsistent results in chapter 3 and chapter 4 may due to the detrimental effect of DMSO to neurons and glia which may conceal the effect of the injury to the cells. This is an unexpected result, given that the DMSO concentration utilized has been used in other investigations without detrimental effect being noted (Chuckowree et al., 2018, Clark et al.,

2018). Further studies investigating various concentrations of DMSO, as well as potential use of other vehicles for dissolving the epothilone would be required to definitively determine effects.

Second, several potential mechanisms may underly the sex dependent axonal pathology noted in the current study. In Dollé and colleagues' study, after culturing female and male axon tracts from rat embryos and human iPSC neurons, female axons were consistently smaller with fewer microtubules than male axons (Dollé et al., 2018). These structural differences may put the microtubules in female axons at greater risk of failure during trauma (Dollé et al., 2018). Additionally, the increased presence and absence of testosterone may play a role in sex dependent axonal pathology, as Pasaresi and colleagues have reported that manipulating of testosterone exposure in rats had significant effects on the size of axon (Pesaresi et al., 2015). Furthermore, microglial activation may also play a role. Microglia have been suggested with removing the cellular debris in order to help the recovery process after brain injury (Neumann et al., 2009). In the current study, the percentage of Iba1 was higher in female cohorts compared with male cohorts, which might help female injured axons more quickly recovery. Mitochondrial dysfunction has also been associated with axonal damage (Neumann et al., 2009). Gaignard and colleagues reported that mitochondria in female rodent brains had lower oxidative stress compared with those from males, which are associated with greater expression and activity of antioxidant enzymes (Gaignard et al., 2015). Thus, sex dependent mitochondrial responses may be another reason for sex dependent difference in axonal damage.

A potential caveat for this data, is that brain swelling was not evaluated in current studies. Using the same injury model as our study in rats, Schneider and colleagues found that the mild injury induced significant higher brain edema measured by MRI imaging at both 4 and 8 hrs after injury, however this difference was resolved at 12 hrs after injury (Schneider et al., 2002). Moreover, Maegele and colleagues observed that mild to moderate injury produced pronounced development of vasogenic edema and cytotoxic edema in brains at 48 hrs after injury detected by MRI imaging, however, at 7 days post injury, this edema was also completed normalized (Maegele et al., 2015). Therefore, at the timepoints (1 week and 4 weeks post injury) when the analysis was performed in the current study, it is likely that injury induced brain swelling may be completely resolved, however, future studies using MRI would be needed to confirm this.

Furthermore, in the current study, I haven't calculated the essential number of animals to get the powerful for parametric tests before the experiments. Therefore, one thing needs to keep

in mind is that, the difference between some groups in our study was not statistically different, which may due to the limited animal numbers used. Therefore, future follow up studies may be strengthened by additional animals per group depending upon outcomes. Following the pilot study completed in the current thesis I determined that n=17 per group of animals are required to achieve an appropriately powered preclinical trial of Epothilone D with DMSO as the vehicle control. Given the novelty of the data, future larger experimental groups are required to validate the potential of Epothilone D at treating axonal degeneration.

In summary, the findings in the current thesis suggest that the three risk factors, age, sex and additional injury, did not overtly affect the duration of apnoea, the period of unconsciousness, and the pyramidal population in the neocortex. However, these factors differentially affected the glial and axonal responses after injury (summarized in Fig 6.1, Fig 6.2, Fig 6.3 and Fig 6.4). Thus, it suggests that the age and sex of the person affected, as well as the potential repetitive nature of the injury, should be taken into consideration when making guidelines for mTBI patients.

6.1. INFLUENCE OF AGE AND SEX IN OTHER NEUROLOGICAL DISEASE MODELS

The influence of age and sex has been reported in other neurological diseases. In stroke, it has been reported that ageing related white matter change is associated with higher risk for stroke (Oksala et al., 2009). Additionally, aged animals in ischemic stroke models showed worse outcomes including earlier BBB disruption, higher neuronal degeneration and worse functional outcomes (Rosen et al., 2005, Dinapoli et al., 2006, DiNapoli et al., 2008). Furthermore, the aged are more vulnerable to ischemic injury with more severe white matter atrophy, higher axonal degeneration and worse long-term sensorimotor and cognitive deficits after stroke (Rosenzweig and Carmichael, 2013, Suenaga et al., 2015). In neurodegenerative diseases, it is reported that the ageing of white matter change is strongly correlated with the risk of neurodegenerative diseases, with decreased white matter volume, increased white matter lesions, impaired white matter integrity and functional network alterations in Parkinson's disease (Watanabe et al., 2013), and white matter ischemia and subsequent white matter damage in Alzheimer's disease (Liu et al., 2017). Moreover, ageing is the most significant factor influencing the clinical presentation and disease course and progression of Parkinson's disease (Hindle, 2010, Wyss-Coray, 2016).

The influence of sex on the outcomes after mTBI has been reported. Sex influences the play behaviour after mTBI, with females being less engaged in play activities than males (Mychasiuk et al., 2014). Additionally, females have been found to be more active and more

anxious, with persisting deficits in spatial learning compared to male rodents after mTBI (Wirth et al., 2017). Moreover, in Russell and colleagues' study, they reported that, at 7 to 10 days after mTBI in mice, injury decreased c-Fos-immunoreactivity in non-neuroendocrine corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus (PVN) in females but not in males, which indicates a sex-dependent link to stress dysregulation of preautonomic neurons in females and potentially a sex dependent manner of mTBI altered hypothalamic-pituitary-adrenal (HPA) axis (Russell et al., 2018).

The incidence of stroke is sex dependent with females having a lower incidence compared to males at a younger age. Moreover, younger females are more resilient to ischemic brain injury compared to younger males rodents (Vannucci et al., 2001). This trend reverses at a later age, and overall females suffer more stroke events than men and are less likely to recover (Reeves et al., 2008). With regard to Alzheimer's disease, it is more prevalent in females above age 65 years (Irvine et al., 2012), and the cognitive deterioration is worse in females (Plassman et al., 2011, Irvine et al., 2012). As to Amyotrophic lateral sclerosis, it is more prevalent in males, with increasing risk in post-menopausal females (del Aguila et al., 2003, McCombe and Henderson, 2010). In regards to multiple sclerosis, it is more common in females, and is faster progressing in males (Confavreux et al., 2003, Voskuhl and Gold, 2012). Finally, as to Parkinson's disease, it is more prevalent in males, with a slower rate of functional decline in females (Baldereschi et al., 2000, Elbaz et al., 2002).

6.2. MEASUREMENTS USED IN THE CURRENT STUDY

In the current study, we have used various measurements for axonal degeneration in the internal capsule white matter tracts: The percentage area of axonal degeneration indicates the percentage area taken by axonal bulbs in the internal capsule; The relative number of the axonal fragments in the internal capsule indicates the actual number of dissociated, non-continuous axonal fragments relative to sham operated mice; The relative the size of the bulbs indicates the size of the axonal fragments. As reported in this thesis these three measurements are not always consistently changed, with individual changes sometimes occurring. Take results in Chapter 3 for example, there were increased percentage of area and higher numbers of axonal fragments after injury at 1 week post injury in 1.5 months mice, however, there was no significant changes in the size of axonal fragments. This suggests there is more axonal degeneration and more fragments but not the size of the fragment in young animals at 1 week after injury. Our finding is similar to Mannix and colleagues' results, in which the injury exacerbated white matter loss in adult mice but not in adolescent animals at a late time point after repetitive mTBI (Mannix et al., 2017). Furthermore, Pernici and

colleagues observed that a subset of undulating axons in the external capsule, caused by moderate TBI, returned back to baseline morphology and previously noted varicosities disappeared (Pernici et al., 2019). In the current study, axonal degeneration in the internal capsule was observed at 1 week post injury but not at 4 weeks post injury in mice injured at 1.5 months, one possible explanation might be that the degenerated axons at 1 week post injury returned back to normal at 4 weeks post injury in 1.5 months mice, which may suggest the plasticity of the young brain to mTBI. Additionally, the increased number of fragments in the young at 1 week after injury suggests there were higher axonal fragments numbers, together with the increased percentage area of fragments, which may indicate the vulnerability of the young patient to mTBI at short-term after injury (Emery et al., 2016, Jones et al., 2018, Lele et al., 2019).

In the present study, we investigated axonal degeneration in the internal capsule white matter tracts, as this is a large white matter tract located directly under the injury site. The corpus callosum was considered, but ultimately not used for analysis as we did not observe overt axonal degeneration in this region (data not shown). In other studies, exacerbated white matter tract loss has been reported after repetitive mTBI in the adult mice (Mannix et al., 2017). Additionally, it has also been reported that there is higher rates of axonal degeneration in the corpus callosum after repetitive mTBI in aged compared to young brains (Ferguson et al., 2017b, Mouzon et al., 2018b). In the current study, we used the Thy1-YFPH mice to visualize morphological changes in axons. We haven't used the immunolabelling of APP in our study as our preliminary investigations did not reveal overt axonal degeneration by APP immunolabelling. This is probably because the accumulated APP in the axons has been degraded and cleavage by microglia at the time points investigated (Stone et al., 2002, Iwata et al., 2002, Chen et al., 2017). Apart from this, our findings are similar to previous studies (Mouzon et al., 2018b, Collins et al., 2019). Collins and colleagues reported that APP accumulated in the neuronal processes within the cortex, and within axonal swellings and bulbs in the corpus callosum at 24 hrs after mild to moderate TBI in adult mice (Collins et al., 2019). Moreover, Mouzon and colleagues found that APP immunoreactive axonal profiles were observed in the corpus callosum at 24 hours after rmTBI in adult and aged brain injured mice (Mouzon et al., 2018b).

Measures of the average thickness of the neocortex at the injured area indicate whether the injury caused a major tissue loss or even cavitation. This has been widely observed in the brain after moderate and severe TBI (Hakon et al., 2015, Ouyang et al., 2017). However, minor neuronal apoptosis and death may not be identified by this measurement. Further studies could use specific neuronal degeneration markers to identify this, for example NeuN or Fluoro-

jade B (FJB). Additionally, MRI could be used as a tool to measure cortical changes *in vivo*. Scholz and colleagues reported that MRI imaging yields evidence of structural changes, they found that the hippocampus, frontal cortex and amygdala are larger in rotarod-trained mice compared to untrained controls. Moreover, the cerebellum and white matter in the corpus callosum underlying the primary motor cortex are smaller after rotarod training (Scholz et al., 2015). Furthermore, Hammelrath and colleagues reported that the mouse brain volume was almost stable at three weeks of age, but thickness of cortex kept decreasing continuously with maximal changes during the first three months of mice age detected by *in vivo* MRI (Hammelrath et al., 2016). Thus, MRI could be a potential tool to investigate different timepoints after mTBI in mice in the future. Difference of cutting angle during brain slicing may exist among different animals, which may influence the results of cortical thickness, therefore, other methods could be used, such as MRI imaging which could perform *in vivo* imaging and use less animals.

We did not find any significant dendritic spines changes after injury. By using Golgi-Cox staining, Gao and Xiang reported that mTBI significantly decreased the density of mushroom and filopodia spines, and increased stubby spines in mice cortex at 3 days after injury (Gao and Chen, 2011). Furthermore, by applying *in vivo* two photon imaging in the mouse brain, Kislin and colleagues found that the dendritic segments in the injured cortex contained stable spines as well as undergoing spine gain and loss over 2 weeks after the injury (Kislin et al., 2017). Therefore, in future studies, using other methods for detecting the dendritic spines are warranted, including Golgi-Cox staining as well as using *in vivo* two photon imaging.

6.3. STRENGTHS OF CURRENT STUDY

Although, alternate investigation and analysis protocols can always be applied, there are numerous strengths to the current study. Firstly, we used various aged animals of a single species in a single study. Moreover, we applied lateral fluid percussion injury in a mouse model. This model has been widely used to mimic mTBI, as it induces both diffuse and local brain injury, closely resembling human mild brain injury. Additionally, this model is reliable and replicable. As it has been reported that the procedure of surgery itself may induce a glial response (Lagraoui et al., 2012, Chuckowree et al., 2018), we included sham operated mice in our study, which enabled us to eliminate the influence of the surgery itself from our interpretation. Furthermore, we investigated cellular and subcellular responses at two timepoints after injury, which enable us to investigate both the short and long term response, as well as the progress and evolution of the response after injury. Moreover, we used Thy1-YFPH transgenic mice, which enable us to visualize the neuronal cell body as well as axons

and dendrites. Furthermore, we applied various measurements, including the percentage area of axonal degeneration, the average size and the relative number of axonal fragments, to investigate how the internal capsule white matter tracts response to mTBI. In the current study, rather than delivering the epothilone D prior to or immediately after injury, we administered the epothilone D at 24 hrs after injury in order to match clinical drug treatment timepoints. Additionally, unlike applying a drug in a single aged cohort, we used both young and adult mice animals to investigate whether there is age dependent effect of epothilone D treatment. As the influence of sex in the context of mTBI is less well studied, we investigated the cellular reactions after mTBI in both male and female Thy-YFPH mice. For repetitive injuries, how the number of injuries and the time interval between injuries influence the outcomes after repetitive mTBI not well investigated. We therefore, investigated how additional injury affects the cellular reaction.

6.4. FUTURE DIRECTIONS AND LIMITATIONS

Despite these strengths, there are still some shortcomings in the present study, that should be acknowledged and considered when interpreting our findings. Firstly, we only used a single mTBI injury model in our study. While the LFPI model has a number of advantages, it is not without its disadvantages. The injury process includes anesthesia and craniotomy, which has been suggested to affect the cellular and subcellular reactions, and they are seldom observed in mTBI patients (Lagraoui et al., 2012, Cole et al., 2011, Wu et al., 2019, Rowe et al., 2013). There are other mTBI models, such as the weight drop injury model, cortical controlled injury (CCI) model, and blast injury model, however, each has advantages and disadvantages. In regards to the weight drop injury model, the advantages include easy manipulation, high reproducibility, well-studied neuropathology and the ability to represent graded forms of diffuse TBI (Viano et al., 2012). The disadvantages are the possibility of re-hits to the head or body of the animals (Mychasiuk et al., 2014), difficulty of skull fixation (Viano et al., 2012) and the potentially confounding effects of anaesthesia during the injury procedure (Yurdakoc et al., 2008). With regard to CCI model, the advantages include the ability to directly control physical damage, reliable deficits (Lagraoui et al., 2012), and no rebound concussive events (as seen in weight drop model) (Mychasiuk et al., 2014), while a disadvantage of the model is the need to perform craniotomies (as seen in fluid percussion injury model). As to the blast injury model, the advantages are its high clinical relevance to returning veterans and also its high reproducibility. However, disadvantages of the blast injury model include the high price of the device and the need for an extensive experimental set up, as well as complications associated with the model itself, including intra-parenchymal hemorrhage along the trajectory of the first impact (Sato et al., 2014). Thus, no model is perfect at replicating the heterogeneous nature

of mTBI in the human population, and new models free from anesthesia and craniotomy are needed for use alongside the current LFPI model.

Additionally, even though we induced the injury in the middle between bregma and lambda skull suture, we cannot be sure that the site that the injury was delivered to represents the exact same anatomic site across the lifespan due to the different sizes of the brains at each age point. It has been reported by Rowe and colleagues, that different sized trephines were used to attempt to circumvent the potential confounder of age animal at injury (Rowe et al., 2016). This could be considered for future studies.

In the current study, we investigated both 1 week and 4 weeks post injury time points. However, Mouzon and colleagues reported that repetitive mTBI induced stronger astrogliosis response and microgliosis response in the corpus callosum at 24 hrs after injury in aged mice. Additionally, the injury also caused higher axonal degeneration within the corpus callosum in both adult and aged mice (Mouzon et al., 2018b). Furthermore, Monzou and colleagues also reported that there was significantly higher axonal degeneration in the corpus callosum, higher persisting astrogliosis in the corpus callosum, and higher microgliosis in the corpus callosum at 24 months after rmTBI in adult mice (Mouzon et al., 2018c). Thus, investigation of cellular changes at less than 1 week and longer than 4 weeks post injury are warranted in future studies.

In the current study, we investigated the mice at 1.5, 3 and 12 months of age. In Mannix and colleagues' study, repetitive mTBI was conducted in adolescent mice (5 weeks) and adult mice (4 months). They found that injured adolescent mice had longer loss of consciousness compared to injured adult mice and the effect of injury was worse in adolescent compared to in adult mice with regard to spatial memory (Mannix et al., 2017). In Sandhir and colleagues' study, brain injury was performed in adult and aged mice (22-24 months). In the adult brain, they reported increased expression of miR-21 after injury, with the maximum increase at 24 hrs followed by a gradual decrease, returning to baseline at 7 days post injury. In contrast, miR-21 showed no injury response in aged mice (Sandhir et al., 2014). Furthermore, in Ritzel and colleagues' study moderate TBI was performed in young (3 months) and aged (18 months) mice. They reported that aged mice demonstrated more severe deficits in forelimb grip strength, balance, and motor coordination and anxiety like behaviour, as well as more robust microglial proliferation at 3 days after injury (Ritzel et al., 2019). Therefore, to better understand the influence of age on outcomes after mTBI in a mouse model, future studies investigating on mice across the entire lifespan are needed.

Additionally, as significant cellular changes have been observed after a single injury in female mice in the current study, it would be intriguing in future studies to investigate how the age at injury, treatment with EpoD, and additional injury also affects outcomes in female mice. Further studies should take the sex hormone level in the females at the time of injury into consideration, as female hormones have been suggested to have potentially neuroprotective effects in brain injury (Al-Tarrah et al., 2017). In Khaksari and colleagues' study, brain injury was performed in ovariectomized female rats and extra estrogen was administered following TBI. At 24 hrs after injury, they reported that the estrogen reduced injury induced brain edema, and the edema was attenuated by antagonist of estrogen receptor. Moreover, estrogen reduced the level of IL-1β, IL-6, and TNF-α in the injured brain, and the anti-inflammatory effect of estrogen was decreased by the antagonist of estrogen receptor. Estrogen also increased IL-10 in the injured brain and an antagonist of estrogen receptor resulted in a reduction in proinflammatory cytokines (Khaksari et al., 2015). In order to understand the underlying mechanisms on how sex influence the inflammation process after mTBI, inflammatory factors, such as IL-6, IL-10, TNFα, and C-C Motif Chemokine Ligand 2 (CCL2), cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are needed in future studies.

In the current investigation of repetitive brain injury, we did not include sham operated mice. For this reason, these studies can only, at this stage be presented as preliminary, or 'pilot', which makes our study less powerful. The process of the surgery itself has been reported with the potential of disrupting the cortex and initiating glial responses (Lagraoui et al., 2012, Chuckowree et al., 2018). The repetitive mTBI includes multiple sham process, which could influence the cellular response. Therefore, sham operated mice should be included in the future.

It is suggested that demyelination and remyelination are important components of white matter degeneration in mTBI with axonal injury, in which, oligodendrocyte plays an important role (Mierzwa et al., 2015). Mierzwa and colleagues reported that both oligodendrocyte apoptosis as well as increased newly differentiated oligodendrocyte were observed at 3 days after mTBI in mice (Mierzwa et al., 2015). In the current study, significant increase in the degeneration of axons in the internal capsule has been observed after mTBI, therefore, investigating the population of oligodendrocyte, including mature and immature oligodendrocyte, may provide informative results.

Changes in behavioural and cognitive function has been widely observed as acute and chronic responses to mTBI (Mouzon et al., 2018b, Paterno et al., 2018, Zhang et al., 2019). Using the

same injury model, at 2 days post injury in rats, injury impairs function on novel object recognition test, with injured rats spent less time with the novel object (Jaiswal et al., 2019). At 3 days post injury in the rat, there was increased cognitive deficits in the water maze, but without difference in beam task, elevated plus maze or open field tests (Wright et al., 2016). At 7 days post injury in mice, injured animals had higher freezing rate in conditioned fear response compared to sham operated animals (Smith et al., 2012). Therefore, future studies performing behavioural tests including novel object recognition test, cognitive test and conditioned fear response tests are warranted in future studies.

In the current study, only a single neuronal population was investigated, other neuronal populations including interneurons would be influenced by the injury and should be investigated in future studies. Brizuela and colleagues have reported that calretinin interneurons in injured mice had a reduction in mean dendrite length and a reduction in the number of secondary dendrites comparted to those in the sham-injured controls by 7 days post-injury, and these alterations evolved over a 28 days period (Brizuela et al., 2017). Additionally, Vascak and colleagues reported that parvalbumin interneurons were detected across the somatosensory cortex in layers 2-6 at 3 hrs post mTBI (Vascak et al., 2018).

A limitation of the present study, may be the utilisation of the percentage area of positive GFAP and Iba1 in the neocortex as a measure of the activation of astrocytes and microglia. Previously, GFAP has been used to imply astrocytic activation (Liu et al., 2014, Yu et al., 2016), similarly, Iba-1 has been used as a marker for the microglial activation in the brains in animal studies (Fukuda et al., 2015, Gao et al., 2015, Woodcock and Morganti-Kossmann, 2015). However, it has also been suggested that even though Iba1 expression is increased following mTBI, it is expressed not only in activated microglia but also in resting microglia as well as in circulating macrophages in the brain (Walker and Lue, 2015). Classifying microglial morphology (such as resting microglia or activated microglia) has been suggested to be a good way to imply the states of microglia (Ahmed et al., 2007). This method has been used in Chapter 3 (I newly added the data as Fig 3.4 C, D, E), from which, the results from classifying the morphology of microglial were similar to the results from measuring the percentage area of positive Iba1. In future, applying this microglial morphology analysis to other Chapters to confirm the microglial activation results is needed. Furthermore, applying a nuclear counterstain, such as DAPI, would also allow assessment of cell density independence of immunoreactivity. In addition, *in vivo* imaging for transgenic mice where astrocytes or microglia are labelled may provide useful information (Guo et al., 2017, Hierro-Bujalance et al., 2018).

6.5. CONCLUSIONS

There are many factors that could influence outcomes after mild traumatic brain injury, however, it is still unclear how the age at injury, sex, as well as number of injuries and interval between injuries influence the cellular and subcellular reactions after mild traumatic brain injury. This thesis demonstrated that glial activation after mTBI was age dependent, with aged animals having higher glial activation. Axonal degeneration after mTBI was also age dependent, with adult and aged brains showing persisting degeneration that was not in young brains (summarized in Fig 6.1). Furthermore, findings of this thesis indicated the age dependent axonal and glial responses after mTBI. Additionally, this thesis demonstrated that the treatment potential of epothilone D, as a modifier of axonal degeneration, was also age dependent, and was found to be ineffective in young mice and detrimental in adult mice (summarized in Fig 6.2). Female injured animals showed alleviated cellular reactions compared to males, with increased and earlier resolving of axonal pathology after injury (summarized in Fig 6.3). Additionally, a second injury at 48 hrs after the initial injury significantly increased the cellular reaction and subcellular changes in comparison to a single brain injury, with increased glial responses and higher axonal degeneration observed (summarized in Fig 6.4). Overall this thesis provides important insight into how age at injury, sex, number of injuries and interval between injuries differentially affected neuronal and glial reactions to mild traumatic brain injury. Specific cellular and subcellular responses were found to differentially characterize pathological changes and recovery processes after mTBI. Thus, this thesis further suggests more attention should be spent on addressing these risk factors when assessing patients and provides further evidence for the development of individualized treatments for mTBI patients.

7. APPENDIX

7.1. Immunohistochemistry solutions

0.3% Triton/PBS

600µL Triton X (Sigma, USA)

200mL 0.01M PBS

4% Paraformaldehyde (PFA)

40g PFA (Sigma, USA)

100mL 9% NaCl

400mL Na2HPO4

500mL NaH2PO4.2H2O

Heat while stirring in a fume hood.

7.2. Normality tests results

Table 1. aging data set (Chapter 3) normality results

Measurements	Graph in thesis	Pass normality test
cortical thickness	Fig 3.1 A	yes
YFP+ cell density	Fig 3.1 B	no
YFP+ soma size	Fig 3.1 C	no
GFAP area	Fig 3.1 F	yes
iba-1 area	Fig 3.1 G	no
axon percentage of area	Fig 3.1 H	no
axon fragment number	Fig 3.1 I	no
axon fragment size	Fig 3.1 J	yes
total spine density	Fig 3.1 K	yes
thin spine density	Fig 3.1 L	no
stubby spine density	Fig 3.1 M	yes
mushroom spine density	Fig 3.1 N	yes

Table 2. aging with injury data set (Chapter 3) normality results

Measurements	Graph in thesis	Pass normality test
apnoea	Fig 3.2 B	yes
righting reflex time	Fig 3.2 C	yes
cortical thickness	Fig 3.2 D	yes
YFP+ cell density	Fig 3.2 E	no
YFP+ soma size	Fig 3.2 F	yes
GFAP area	Fig 3.3 B	no
iba-1 area	Fig 3.4 B	no
resting microglia number	Fig 3.4 D	no
activated microglia number	Fig 3.4 E	no
axon percentage of area	Fig 3.5 B	no
axon fragment number	Fig 3.5 C	no
axon fragment size	Fig 3.5 D	no
total spine density	Fig 3.6 B	yes
thin spine density	Fig 3.6 C	no
stubby spine density	Fig 3.6 D	yes
mushroom spine density	Fig 3.6 E	yes

Table 3. treatment sham data set (Chapter 4) normality results

Measurements	Graph in thesis	Pass normality test
pulse	Fig 4.2 A	yes
apnoea	Fig 4.2 B	yes
righting reflex time	Fig 4.2 C	no
cortical thickness	Fig 4.2 F	yes
YFP+ cell density	Fig 4.2 H	no
YFP+ soma size	Fig 4.2 J	no
GFAP area	Fig 4.3 D	yes
iba-1 area	Fig 4.3 F	yes
total spine density	Fig 4.5 B	no
thin spine density	Fig 4.5 D	no
stubby spine density	Fig 4.5 F	no
mushroom spine density	Fig 4.5 H	yes

Table 4. treatment injury data set (Chapter 4) normality results

Measurements	Graph in thesis	Pass normality test
cortical thickness	Fig 4.2 E	yes
YFP+ cell density	Fig 4.2 G	yes
YFP+ soma size	Fig 4.2 I	yes
GFAP area	Fig 4.3 C	yes
iba-1 area	Fig 4.3 E	no
axon percentage of area	Fig 4.4 B	yes
axon fragment number	Fig 4.4 C	yes
axon fragment size	Fig 4.4 D	yes
total spine density	Fig 4.5 A	yes
thin spine density	Fig 4.5 C	yes
stubby spine density	Fig 4.5 E	yes
mushroom spine density	Fig 4.5 G	yes

Table 5. influence of sex after injury data set (Chapter 5) normality results

Measurements	Graph in thesis	Pass normality test
apnoea	Fig 5.1 B	no
righting reflex time	Fig 5.1 C	no
cortical thickness	Fig 5.1 D	yes
YFP+ cell density	Fig 5.1 E	yes
YFP+ soma size	Fig 5.1 F	yes
GFAP area	Fig 5.2 C	no
iba-1 area	Fig 5.2 D	no
axon percentage of area	Fig 5.3 B	no
axon fragment number	Fig 5.3 C	no
axon fragment size	Fig 5.3 D	yes
total spine density	Fig 5.4 A	yes
thin spine density	Fig 5.4 B	no
stubby spine density	Fig 5.4 C	no
mushroom spine density	Fig 5.4 D	yes

Table 6. influence of an additional injury data set (Chapter 5) normality results

Measurements	Graph in thesis	Pass normality test
apnoea	Fig 5.5 A	yes
righting reflex time	Fig 5.5 B	yes
cortical thickness	Fig 5.5 C	yes
YFP+ cell density	Fig 5.5 D	yes
YFP+ soma size	Fig 5.5 E	yes
GFAP area	Fig 5.6 B	no
iba-1 area	Fig 5.6 C	no
axon percentage of area	Fig 5.7 B	yes
axon fragment number	Fig 5.7 C	yes
axon fragment size	Fig 5.7 D	yes
total spine density	Fig 5.8 A	yes
thin spine density	Fig 5.8 B	yes
stubby spine density	Fig 5.8 C	no
mushroom spine density	Fig 5.8 D	no

8. REFERENCES

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