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Axon Initial Segment Integrity in Aging and Traumatic Brain Injury

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AXON INITIAL SEGMENT INTEGRITY IN AGING AND TRAUMATIC BRAIN INJURY

A Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

AIS	axon initial segment
AP	action potential
AnkG	ankyrinG
ANOVA	analysis of variance
APP	β-amyloid precursor protein
ATF-3	activating transcription factor 3
atm	atmospheres
BBB	blood brain barrier
Ca2+	calcium ion
CAM	cell adhesion molecule
caspr	contactin-associated protein
cFPI	central fluid percussion injury
CMSP	calpain-mediated spectrin proteolysis
CNS	central nervous system
Da	Dalton
DAI	diffuse axonal injury
DTI	diffusion tensor imaging
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
ED	emergency department
EM	electron microscopy
GABA	Gamma-Aminobutyric Acid
HRP	horseradish peroxidase
hr	hour
ICU	Intensive Care Unit
IL	Interleukin
Kv	voltage-gated potassium channel
LOC	loss of consciousness
LM	light microscopy
LPS	lipopolysaccharides
min	minute
mL	milliliter
mPTP	mitochondrial permeability transition pore
MRI	magnetic resonance imaging
Na+	sodium ion
Nav	voltage-gated sodium channel
NF-155	neurofascin-155
NF-186	neurofascin-186
NOR	Node of Ranvier
NOD	Nicotinamide Adenine Dinucleotide
NrCAM	neuronal cell adhesion molecule
OCT	Optimal Cutting Temperature™
PBS	phosphate buffered saline
PNS	peripheral nervous system

RER	rough endoplasmic reticulum
RTA	road traffic accident
SCWM	subcortical white matter
SER	smooth endoplasmic reticulum
SERCA	SER Ca2+-ATPase
TAI	traumatic axonal injury
TBI	traumatic brain injury
YFP	yellow fluorescent protein
μm	micrometer

ABSTRACT

AXON INITIAL SEGMENT INTEGRITY IN AGING AND TRAUMATIC BRAIN INJURY

By Mazen Gouda, B. S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master's of Science at Virginia Commonwealth University

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According to the Center for Disease Control's (CDC) report to the Congress, there are 2.2 million emergency department visits; 80,000 hospitalizations; and 50,000 deaths each year due to traumatic brain injury. Adults 65 years and older account substantially for the majority of the hospitalization and deaths. Over 70% of the traumatic brain injuries of the older adults are classified as mild to moderate; however, even with these milder injuries, older adults present with a significantly higher morbidity and mortality compared to all other age groups (LeBlanc et al., 2006). With that in mind, it seems essential to develop a deeper understanding of the causes behind higher mortality and morbidity of traumatic brain injury in the elder population. It is well documented that increased age is accompanied by increased CNS inflammation. Recently, our laboratory showed that inflammation drives brain pathology. Specifically, we reported that the axon initial segment of cortical neurons was structurally and functionally compromised in an inflamed CNS environment. With this in mind, we proposed that age-related inflammation

predisposes that brain to exacerbated pathologic consequence. To test this hypothesis, we administered a mild to moderate central fluid percussion brain injury in aged and young adult mice. Using immunocytochemical labeling against the axon initial segment protein ankyrinG combined with laser scanning confocal microscopy, we quantitatively compared axon initial segment number and length between age groups and within age groups with and without injury. Additionally, we also quantified global axonal pathology by immunolabeling for amyloid precursor protein (APP) positive swelling as an indicator of compromised axonal transport. We proposed that ankyrinG labeling will be both reduced in the aged injured mice compared against aged uninjured, young adult injured and young adult non-injured. We observed a significant increase in APP accumulations due to injury independent of aging, and due to aging independent of injury. No significant changes in the effect of injury between young and aged injured mice were observed. Although AIS length was not altered between age groups following injury, our results demonstrate that the elderly population presents with significantly shorter initial segments. The consequence of this shortening is not clear but may reflect compensatory changes in the brain to maintain homeostasis.

CHAPTER ONE

INTRODUCTION

1.1 Traumatic Brain Injury Epidemiology

According to the Center for Disease Control's (CDC) report to Congress, there are 2.2 million emergency department (ED) visits, 80,000 hospitalizations, and 50,000 deaths each year due to traumatic brain injury (TBI) culminating in an annual cost of $76 billion (CDC, 2013). Adults 65 years and older account for the majority of the hospitalization and deaths. Over 70% of traumatic brain injuries of the older adults are classified as mild to moderate; however, even with these milder injuries, older adults present with a significantly higher morbidity and mortality compared to all other age groups (LeBlanc et al., 2006; de Guise et al., 2014).

The age group with the greatest number of TBI-related ED visits, hospitalization and deaths is the group of 65 years of age and older (Taylor et al., 2017; Faul et al., 2010; Ramanathan et al., 2012). TBI among older adults is distinct from those of younger individuals and requires a unique approach to clinical management and research (Gardner et al., 2018). While TBI occurrence in younger individuals is more prevalent in men resulting from motor vehicle accidents, in older adults TBI is more prevalent in women and results from falls (Taylor et al., 2017; Cuthbert et al., 2015; Leblanc et al., 2006; Harvey et al., 2012; Coronado et al., 2011; Dams-O'Connor et al., 2013). It has been reported that with aging, the brain's white matter and vasculature become more susceptible to injury (Liu et al., 2017; Ikonomovic et al., 2017). Additionally, injury response mechanisms such as autophagy are lowered (Raj et al., 2017). This is accompanied with an increase of pre-existing neurological or systemic comorbidities (Dams-O'Connor et al., 2016). Hence, older adults with TBI experience higher mortality and morbidity

(Ramanathan et al., 2012; McIntyre et al., 2013; Dams-'Connor et al., 2013; Coronado et al., 2011). This manifests with slower recovery (Mosenthal et al., 2004; Cifu et al., 1996; Christensen et al., 2008; Frankel et al., 2006). Also older adults experience worse functional cognitive and psychological outcomes than younger patients post-TBI (Cuthbert et al., 2015; Mosenthal et al., 2004; Thompson et al., 2012; Stocchetti et al., 2012; Gardner et al., 2018). Still, age and TBI severity have been described as inadequate prognostic markers since some older adults with TBI, ranging from mild to severe, experience good recovery. (Lilley et al., 2018; De Bonis et al., 2010; Taussky et al., 2012; Gardner et al., 2018). Recovery can be complicated by cognitive impairment and comorbidities that precede the TBI incident (McIntyre et al., 2013), and pre-existing conditions are extremely common among older adults with TBI (Garner at al., 2018). For example, pre-injury cardiovascular and endocrine disorders are common in older adults with TBI (Brazinova et al., 2016; Korhonen et al., 2013; Fu et al., 2016; de Guise et al., 2014) perhaps predisposing the elderly victim to poor long term prognosis. Moreover, such pre-existing conditions and history of TBI are risk factors for sustaining a TBI resulting in enhanced vulnerability and exacerbated consequences (Gardner et al., 2018). Furthermore, it has been reported that self-rated poor health in the year preceding TBI is predictive of poor outcome after mild traumatic brain injury (mTBI) (Kristman et al., 2016).

1.2 Traumatic Brain Injury clinical assessment

The Glasgow Coma Scale (GCS) clinical assessment is used to determine TBI severity at the time of initial presentation in which the severity is based on level of consciousness. GCS scores of 13-15 are graded as mild; scores of 9-12 are graded as moderate, and scores $\leq$8 are graded as severe (Parikh et al., 2007). While GCS is the most widely used clinical assessment of

TBI severity, it may lack the ability to accurately assign TBI severity in older adults (Gardner et al., 2018). For instance, pre-existing dementia may result in an abnormal GCS at baseline (Ashby et al., 2016). Additional pre-existing conditions and medication side effects can have the same effect confounding the diagnosis (Papa et al., 2012). Thus, there is a need for objective biomarkers to aid in the diagnosis process (Gardner et al., 2018).

Among adults age 65 years and older admitted to hospital with TBI of any severity, up to 45% present with subdural hematoma apparent on head computed tomography (CT) scan (Hawley et al., 2017). While 5% of younger adults, whose diagnosis was mTBI, were reported to have intracranial trauma, 11-21% of adults ages 65 year with the same mTBI diagnosis, based on GCS of 13-15, were reported to have intracranial trauma (Styrke et al., 2007; Haydel et al., 2000; Stiell et al., 2001; Gardner et al., 2018). Even those with normal GCS (GCS = 15) are at high risk. For example, in one study, 17% of adults, 60 years and older, with a normal GCS, presented with head trauma as indicated by CT scan (Haydel et al., 2000). Another study reported 57% of adults, 60 years and older, had an intracranial hemorrhage as assessed by head CT even though they presented with a normal GCS (Styrke et al., 2007). This higher prevalence of CT evidence of neurotrauma among older patients is hypothesized to be the product of several factors including changes in vasculature and white matter integrity making vessels more susceptible to rupture and white matter more vulnerable to shear injury (Gardner et al., 2018).

Another valuable tool in evaluating post-TBI is structural Magnetic Resonance Imaging (MRI) as it can identify evidence of neurotrauma missed by head CT (Yuh et al., 2013). Other emerging neuroimaging technologies include 7-Tesla MRI and functional MRI (Cen et al., 2016). Also, Positron Emission Tomography (PET) ligands that bind to amyloid-beta, tau and markers of neuroinflammation can play an important role in improving TBI prognosis in older

adults (Yang et al., 2015; Hong et al., 2014; Gardner et al., 2018). However, it has been reported that amyloid and tau neuropathology increase by about 10-15% per decade starting at 60 years of age, and thus it should be used carefully (Braak et al., 1997; Gardner et al., 2018).

The majority of existing rules and validation studies support the routine use of head CT for all patients above 60 years of age presenting with mild TBI even after rapid return to baseline, GCS = 15 (Haydel et al., 2000; Seil et al., 2001; Altman et al., 2015, Mower et al., 2005; Wolf e al., 2014). This includes the American College of Emergency Physicians' recommendation of considering head CT in all patients above 65 years old who present with TBI, even mild injury without loss of consciousness (LOC), and recommends obtaining a head CT in all patients above 60 years of age with TBI and LOC (Jagoda et al., 2008). On the other hand, the Canadian CT Head Rule recognizes ages of 65 years as a high-risk factor for intracranial trauma, and thus in need of neurosurgical intervention among patients presenting with TBI and a GCS of 13–15, regardless of LOC (Steil et al., 2001).

Taking into account the previous criteria for TBI, the number of older adults presenting to the ED and being admitted to neuro-ICUs for management of TBI is expected to continue to increase (Gardner et al., 2019). Hence, there is an urgent need to develop better geriatric-specific prognostic models (Staples et al., 2016; Roe et al., 2015; Gardner et al., 2019). We believe an understanding of the Axon Initial Segment and AnkyrinG, could provide a step toward better understanding of TBI in older adults.

1.3 Traumatic Brain Injury classification can be complex

In mTBI, external forces induce rapid acceleration-deceleration, resulting in subtle CNS dysfunction and/or pathology (Johnson et al., 2013; Povlishock and Katz, 2005). On the other hand, moderate to severe TBI presents with intra-parenchymal mass/focal lesions such as hemorrhage and hematoma formation, contusion and overt cell death/necrosis which are detectable by CT and MRI (Povlishock and Katz, 2005). Moderate to severe TBI causes direct macroscopic tissue damage such as contusion at the impact site (Andriessen et al., 2010; Farkas and Povlishock, 2007; Maxwell et al., 1997; Povlishock et al., 1992). In contrast, in mTBI rapid acceleration-deceleration can cause diffuse microscopic perturbation such as distortion of mechanoporation of neuronal membranes (Povlishock, 1993).

In general, the insult caused by TBI can be classified as primary or secondary; this classification is dependent on when the injury occurs (Adams, 1992; Osborn et al., 2009). Primary injury refers to the initial insult, which affects the brain tissue at the time of injury. Secondary injury is due to cellular and molecular cascades, whose activation is a consequence of the primary injury (Adams, 1992; Weber, 2012). These two classifications of brain injury insults can occur following focal and/or diffuse TBI. It has been reported in previous studies that the age at which humans experience the primary injury has a strong effect on causing a secondary injury (Susman et al., 2002; Wang et al., 2013; Mychasiuk et al., 2015).

1.4 Traumatic Brain Injury can be focal or diffuse

The injury caused by TBI can be focal or diffuse. In a focal TBI injury, the head strikes or is struck by an object causing a penetrating head injury (Gennarelli and Graham, 2005). In a

sense, the brain makes direct contact with a foreign object causing immediate damage to the parenchyma and vasculature at the site of the injury (Lighthall, 1988; Saatman and Duhaime, 2008). This kind of focal injury is associated with contusion that results in a hemorrhage or hematoma within the brain (Granacher et al., 2007). For the purpose of the present study, we focus on diffuse TBI. Diffuse TBI is not limited to the injury site where the direct impact takes place. The injury has a widespread nature which in turn results in less specific symptoms including loss of consciousness, cognitive impairment, and neuropsychiatric issues. (McAllister, 1992; Stocchetti et al., 2012). This may be due to the fact that diffuse TBI causes damage at multiple sites of the brain due to accelerating/decelerating injuries (Gennarelli and Graham, 2005). The accelerating/decelerating injury is a consequence of various tissues of the brain having different densities; still experiencing the same tensile force, a change in the rate of acceleration and deceleration occurs (Graham, 1996; Shepherd, 2004; Osborn et al., 2009). Consequently, different levels of strain are experienced by the neurons and their processes leading to different levels of injuries at these different sites (Smith et al., 2003). As the degree of injury varies, so does the cellular responses (Singleton et al., 202, Baalman et al., 2013, Greer et al. 2013) resulting in uninjured neurons juxtaposed to injured neurons (Povlishock et al., 1983; Adams et al., 1989; McGinn et al., 2009; Greer et al., 2011; 2012; 2013).

1.5 The pathology and prevalence of Diffuse Axonal Injury

Diffuse axonal injury (DAI) has been described as the most prevalent and significant pathological component in mild, moderate, and severe TBI (Povlishock, 1992; Maxwell and Graham, 1997; Smith and Meaney, 2002; Iwata et al., 2004). The level of axonal damage is a major contributing factor to posttraumatic morbidity and mortality, which correlates with the

degree of functional deficits (Adams et al. 1982; Povlishock, 1992; Iwata et al., 2004). In DAI, force induced stress results in discrete areas of scattered axonal disruptions that ultimately progress to disconnection in which the proximal axonal segment remains connected to the neuronal soma and the distal segment progresses to wallerian degeneration (Greer et al., 2011; Lafrenaye et al., 2015). DAI has been characterized among the primary factors underlying unconsciousness and persistent vegetative state following TBI (Wasserman and Koenigsberg, 2007). Using neuroimaging, it has been established that the duration of coma or loss of consciousness correlates to the degree of DAI (Takaoka et al. 2002).

1.6 Simulation of DAI in mTBI

As indicated earlier, DAI possesses a pervasive nature that contributes to subsequent morbidities, while lacking effective treatment; thus, much attention has focused on the creation of experimental models that replicate the DAI in the hope of developing effective therapeutic strategies to attenuate DAI (Sharp et al., 2014). Early DAI models utilized primates to undergo induced traumatic coma via rapid multi-directional acceleration without impact followed by measurements of variables such as comatose period, level of neurological impairment, and location and amount of DAI (Gennarelli et al., 1982). These studies showed that DAI was directly proportional to the degree of injury indicating the role of DAI in TBI morbidity (Gennarelli et al., 1982). Around this same time period, the fluid percussion model was developed, and was used to produce mechanical brain injury in cats to induce elastic deformation and DAI (Sullivan et al., 1976). The fluid percussion model has many advantages over previous models. A consistent injury could be produced via a transient pulse that could be controlled and measured in atmospheres. Even more, it allowed the production of mechanical brain injury

without intraparenchymal or subarachnoid hemorrhage (Sullivan et al., 1976). This was a significant improvement because many of the current mechanical models in TBI caused disruptive lesions involving contusions or hemorrhage; such physical insults do not imitate the progressive axonal injury described in DAI (Povlishock, 1992). Even more, these other pathologies can significantly complicate the evaluation of neuronal alteration or axonal disruption caused by DAI (Povlishock and Katz, 2005).

1.7 Central Fluid Percussion Injury (cFPI) sheds light on the consequences of DAI

The fluid percussion model was utilized in combination with peroxidase-laden gels to label axons and to demonstrate the secondary axotomy produced by DAI (Povlishock et al., 1983). Except in severe cases, the shearing forces in DAI do not cause disconnection of axons (Povlishock et al. 1993; Osborn et al., 2009). In axonal pathology, a secondary injury includes a progressive process involving a series of deleterious molecular cascades (Singleton et al., 2002; Iwata et al., 2004; Greer et al., 2013). This process initiates an immediate increase in intra-axonal calcium at the site of injury that disrupts ionic homeostasis (Fineman et al., 1993; Maxwell et al., 1995; Iwata et al., 2004; Weber et al.; 2012). More specifically, intra-axonal calcium plays a role in axonal pathology. This raises the question of what is the source of calcium? One possibility is that mechanical stretching of the axons leads to extracellular calcium influx through the axolemma (Smith et al., 1999; Weber et al., 2012). Other potential calcium sources are intracellular including smooth endoplasmic reticulum (SER) and damaged mitochondria (Ouardouz et al., 2003; Weber et al., 2012; Nicholls, 2009). It has been reported that DAI results in focal axonal swellings in the axon initial segment (AIS) and the para-AIS/juxtapara-AIS

regions (Greer et al., 2013). Thus, an understanding of the role that the AIS plays may provide insight into the possible pathology of mTBI.

1.8 The Axon Initial Segment

The AIS is the region of the axon that is usually located immediately distal to the soma (**Figure 1.1**), and contains a high density of voltage-gated sodium channels (Kole et al., 2007). This focal concentration of sodium channels highlights the importance of the AIS as it has been established that the AIS is responsible for action potential initiation and modulation (Buffington and Rasband, 2011). The AIS is a conserved structure with a highly developed subaxolemmal cytoskeleton integrated with a unique extracellular matrix (Hedstrom et al., 2007; Ogawa and Rasband, 2008; Rasband, 2011). The AIS plays an important role in regulating neuronal excitability through excitatory and inhibitory synaptic input that determines action potential (AP) generation and modulation (Kole and Stuart, 2012). The AIS regulates neuronal activity through activation and inhibition of the densely packed voltage-gated sodium channels (NaV) (Kole et al., 2008). NaV1.6 channels are located in the distal region of the AIS, and sets the threshold for action potential generation (Kole et al., 2008; Van Wart et al., 2007). However, it has been reported that potassium channels are more localized in the distal region of the AIS, while the NaV in general are present throughout the entire AIS (Inda et al., 2006).

Mice that exhibit compromised clustering of AIS proteins present with ataxia and the inability to initiate action potentials (Zhou et al., 1998). In addition to the voltage-gated sodium channels, these AIS proteins include AnkyrinG (AnkG). AnkG is considered the master organizer of the AIS; it plays a role in the establishment and maintenance of AIS protein clusters

and neuronal polarity (Hedstrom et al., 2008; Jenkins and Bennett, 2001; Grubb and Burrone, 2010). Hence, AnkG is an excellent indicator for assessing AIS integrity (Grubb and Burrone, 2010).

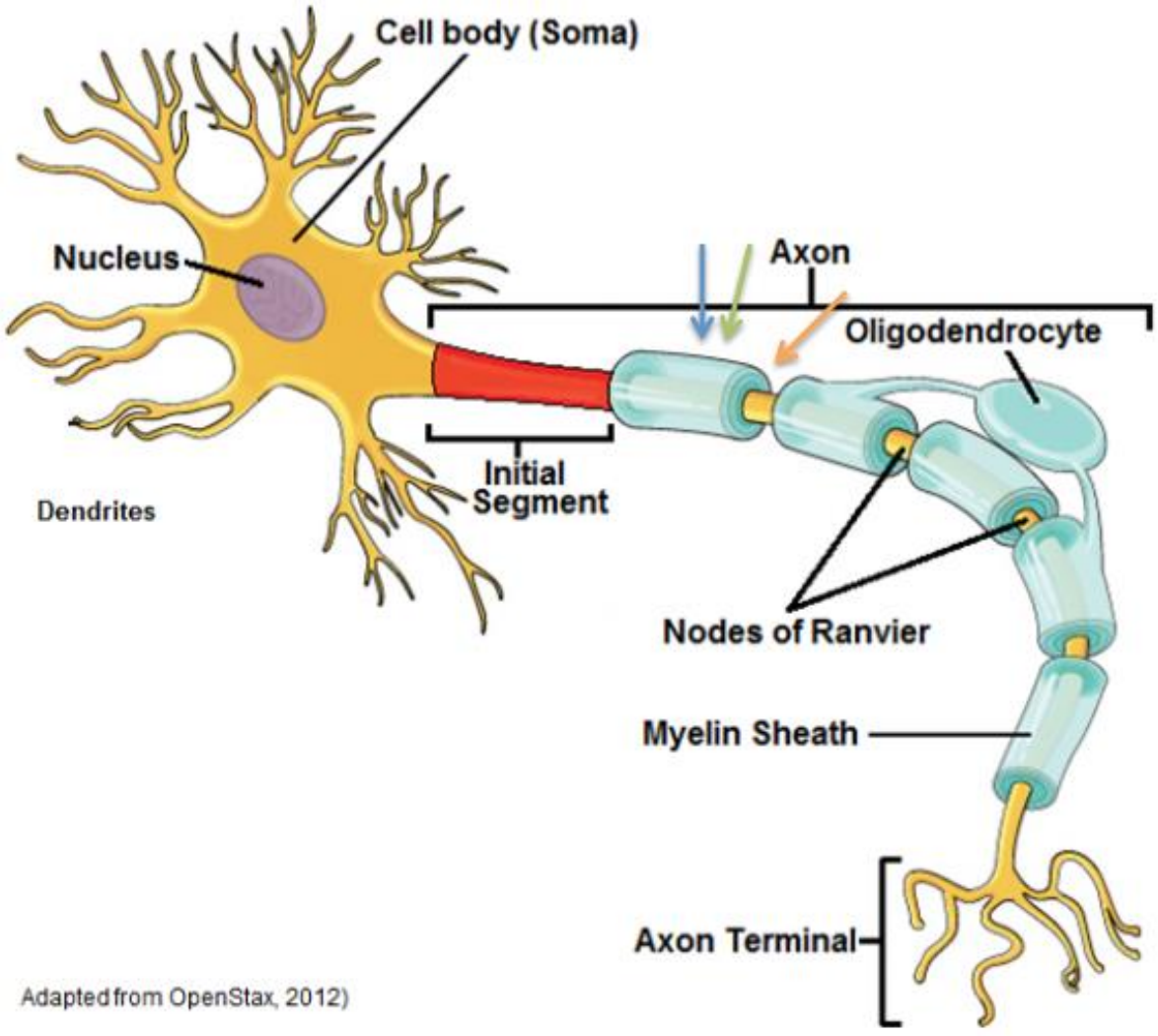
1.9 mTBI effect on the AIS and electrophysiological consequences:-

A previous study analyzed axotomized and non-axotomized neuronal excitability (Greer et al., 2012). The study revealed that the axotomized neurons had a higher action potential (AP) amplitude and a decreased after-hyperpolarization-duration (AHD) when compared to the control mice at both 1 day and 2 days post injury. The study also indicated that neurons with intact axons had similar AP amplitude and AHD to axotomized after 1 day but recovered to normal lower AP amplitude and presented with longer AHD reminiscent of the control group. The study concluded that the non-axotomized axons preserve their capacity for recovery (Greer et al., 2012). Greer et al. (2012) also point out that an increased density of Na+ channels could account for increased AP amplitude, and an increase rate of AP rise since the Na+ channels play a role in creating a depolarizing current. Still, Greer et al. (2012) also pointed out that the same observations could also be due to a decrease in K+ currents, polarizing and hyperpolarizing current. Lastly, these authors ruled out involvement of Na+/K+ in the changes in mTBI due to lack of depolarized resting membrane potential.

While Greer et al. (2013) report that, despite traumatic axonal injury, AnkG immunoreactivity persisted within the axonal cylinder, Vascak et al. (2017) reported that mTBI results in a decrease in AnkG at 2 days post-mTBI within the intact axons in layer V (Vascak et al., 2017). More importantly, Vascak et al. (2017) concluded that this subtle decrease in AIS length in the

Figure 1.1 Axonal domains.

The AIS (red) is directly adjacent to the neuronal soma. The paranode (orange arrow) is the region directly adjacent the node of Ranvier. The juxtaparanode (green arrow) is directly adjacent to the paranode while the internode (blue arrow) is directly adjacent to the juxtaparanode. Adapted from OpenStax, 2012.



Adapted from OpenStax, 2012)

intact axon attenuates AP acceleration. This hints to a compensatory mechanism to the increase in AP amplitude and decrease in AHD in the axotomized neurons described by Greer et al. (2012). Another study reported shortening of the AnkG+ AIS in rats exposed to mTBI, which was accompanied with impaired cognitive function, when compared to the control group at the two weeks point (Baalman et al., 2013). Also, several studies have shown AIS disruption association with multiple diseases that involve an imbalance of network function (Kaphan et al., 2011; Hinman et al., 2013; Hamada and Kole, 2015; Clark et al., 2016; Benusa et al., 2017).

1.10 Neuroinflammation in aging and TBI

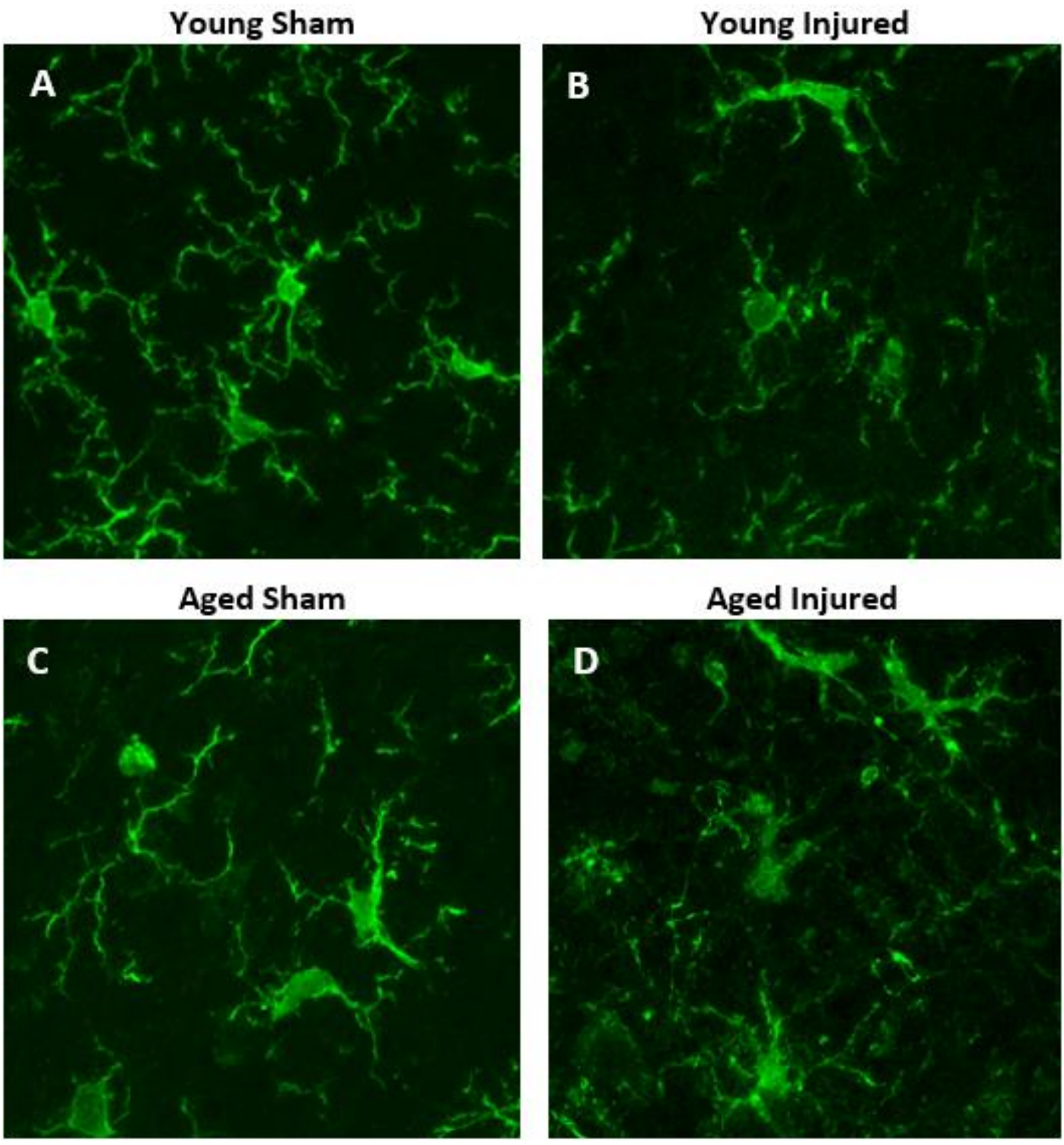
Previous studies have reported that age (Norden et al., 2012) and mTBI (Lafrenaye et al., 2015) independently result in increased CNS inflammation. Microglia is the predominant innate immune cell in the brain, and carries out immunosurveillance of the brain for pathogen invasion, danger signals, cellular debris, apoptotic cells and alterations in neuronal phenotype (Keutzberg, 1996). Previously unpublished assessments of CNS inflammation, shown in **Figure 1.2**, employs microglia morphology in both mild brain injured and sham mice that were 12 (young) and 96 (aged) weeks old. For this assessment we employed a well described morphologic approach (Bilbo et al., 2015) that our lab has previously used (Clark et al., 2016; Benusa et al., 2017) to compare microglial reactivity in layer V of the cortex. As shown in unpublished previously taken images in our lab **Figure 1.2,** microglia from young sham mice exhibited small nuclei with thin, highly branched processes. In contrast, microglia from young mTB injured mice exhibited slightly larger nuclei and their processes were shorter, slightly thicker with fewer processes, which are morphologic characteristics consistent with increased reactivity (Bilbo et al., 2015). Interestingly, microglia from the aged sham mice exhibited morphologies similar to those of the

microglia observed in the young injured mice. In contrast, the microglia in the aged injured mice exhibited morphologies consistent with increased reactivity as their nuclei were larger and their processes were shorter and thicker compared to the young injured and aged sham animals (**Figure 1.2).**

Microglia of a 68 years old human have been reported to be dystrophic and described to have twisted and shortened processes (Streit et al. 2004). It has been reported that while microglia from young mice respond to extracellular ATP, an injury-associated signal, by extension of old processes and formation of new, aged microglia were found to withdraw and even eliminate their existing processes (Demani et al., 2011). Also with aging, microglia exhibit a primed phenotype with an exaggerated and uncontrolled inflammatory response to an immune stimuli (Perry and Holmes, 2014; Niraula et al., 2017)**.**

Figure 1.2 Microglia reactive with injury and aging

Iba1 staining was used to analyze the reactivity of the microglia by assessing its morphology. Images of the neocortex sections between 1.1 mm anterior to the bregma and 2.36 posterior to the bregma. Microglia in young sham mice **(A)** show long thin arms; based on this morphology young microglia in young sham mice are not reactive. However, microglia are reactive in Young injured (**B)**, Aged Sham **(C)**, Aged injured **(D)**; this is indicated by the shorter arms and bulkier cell body **(**Images kindly provided by Dr. Savannah Benusa.)



Aging is associated with an increase in inflammatory signaling, cellular dysfunction and senescence (Fulop et al., 2017). While normally surveying microglia become activated in response to a threat (Colton, 2009), reactive microglia undergo morphological changes, proliferate, and produce pro-inflammatory cytokines (Kreutzberg, 1996). In other cases, the microglia produce anti-inflammatory cytokines to facilitate a return to homeostasis (Colton, 2009).

Chronological aging alters microglia phenotype and sensitivity to injury-induced stimuli (Wong et al., 2013). Studies have reported that the microglia marker known as ionized calcium binding adaptor molecule 1 (Iba1) is chronically elevated in the hippocampus, thalamus and cortex (Sandhir et al., 2008; Kumar et al., 2013). In contrast, other recent studies have reported the number of microglia in aged mice to be lower than in young mice in the cortex (Ritzel et al., 2018; 2019; Sharaf et al., 2013); Sun et al., 2013; Zoller et al., 2018). However, TBI caused a significant increase in microglia counts that is more robust in the old mice than in young injured mice (Ritzel et al., 2019). Aged mice exposed to TBI present with a significantly greater number of peripheral immune cells than young mice exposed to TBI (Ritzel et al., 2019). Prolonged edema and blood brain barrier (BBB) disruption have been reported in aged mice after TBI (Onyszchuck et al., 2008; Timaru-Kast et al., 2012). However, the systemic response to TBI is attenuated with age. Hence, it was concluded that aging alters the proliferative sensitivity of microglia and make the brain more permeable to leukocyte invasion after TBI than their young counterparts (Ritzel et al., 2019). Still, microglia phagocytic activity was significantly increased after TBI in young mice, but was significantly impaired in aged microglia. Hence, debris clearance mechanisms such as phagocytic removal of damaged cells may be highly impaired in old immune cells (Ritzel et al., 2019).

There is an increase in conversion of healthy microglia into dysfunctional dystrophic cells which could impact the brain's ability to maintain and repair itself (Retzel et al., 2019). While TBI-induced pro-inflammatory proliferative potential was increased, basal homeostatic proliferation rates in microglia appeared to be inhibited or impaired (Retzel et al., 2019). Aging has been reported to decrease beta-amyloid uptake in microglia (Nije et al., 2012; Ritzel et al., 2015). This is consistent with reports that rodent and human microglia exhibit a dystrophic phenotype, which is characterized by high granularity, increased activation markers and lipofuscin accumulation with aging (Lopes et al., 2008; Streit et al., 2004; Wong et al., 2013). Microglia dystrophy precedes the spread of tau-dependent neurodegeneration (Streit et al., 2009), which has been linked with AIS pathology. (Chung et al., 2016; Sohn et al., 2016; Hatch et al., 2017)

1.11 Neuroinflammation effect on the AIS

Recently, our lab showed that inflammation drives brain pathology. Specifically, we reported that the axon initial segment (AIS) of cortical neurons was structurally compromised in an inflamed CNS environment (Benusa et al., 2017; Clark et al., 2016). In the chronic neuroinflammation model, EAE, AIS length is reduced in the early stages with a complete loss of AIS protein clustering in the late stages of EAE (Clark et al., 2016). In an acute neuroinflammation model, consequential of intraperitoneal injection of lipopolysaccharide (LPS), AIS counts and length were also reduced (Benusa et al, 2017). AIS protein clustering is also compromised in TBI (Baalman et al., 2013; Greer et al., 2013). However, Vascak et al. (2017) reported that AIS length is reduced in intact axons after mTBI, and point out that this may be a compensatory response to mTBI. However the study was inconclusive on whether the AIS

structural-functional plasticity is adaptive or maladaptive (Vascak et al., 2017). Here, we will determine if TBI in the aged brain, which exhibits an age-dependent inflammatory environment, results in changes of the AIS, which can be an exacerbation of AIS pathology or loss of compensatory mechanism.

We hypothesize that age, independent of injury, will result in an increase of the number of amyloid precursor protein (APP) accumulations in aged sham injured mice as compared to young sham injured mice. We also hypothesize that the number and length of axon initial segments (AISs) will be reduced in the aged sham mice when compared to young sham mice without a reduction in the number of NeuN positive cells. Additionally, we predict that the number of AAP accumulations will be significantly increased in the aged injured mice as compared to aged sham mice and young injured mice. Moreover, we propose that the number and length of the AISs in the aged injured mice will be significantly reduced as compared to the aged sham injured and young injured animals; however, this reduction in AIS number will be in the absence of a loss of NeuN positive cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals:

Nineteen 22- months old, and fifteen 3- months old C57/black6 female mice were purchased from Jackson Laboratories (Bar Harbor, Me, USA) and maintained in the Virginia Commonwealth University Division of Animal Resources vivarium, an AAALAC certified facility. Food and water were provided *ad liberatum.* All housed mice were kept on a 12 hours light/ 12 hours dark cycle and all procedures were conducted according to protocols During the surgical procedures, two 22- months old, aged, and two 3- months old, young, mice died. These mice were excluded from the study reducing the mice number to seventeen aged adult mice, and thirteen young adult mice. The mice weights ranged from 18.3 grams to 33.9 grams.

2.2 Surgical preparation and injury procedure

All surgical procedures were conducted by Dr. John Greer in the laboratory of Dr. John Povlishock as previously described (Greer et al., 2013). Briefly, mice were anesthetized using 4% isoflurane in 100% O_2 for 4 minutes. Anesthesia was maintained using a fitted nose cone with 1-2% isoflurane in 100% O_2. After anesthesia, effect was confirmed by a toe pinch test; the mouse's thigh was shaved for intraoperative physiological monitoring, and the mouse was placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at a constant 37° C during surgery using feedback control via a thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA) that was placed under the mouse, and configured to the monitored rectal temperature. Normal physiological homeostasis (pulse,

respiratory rate, and blood oxygenation) was monitored intraoperatively using pulse oximetry via a thigh sensor (STARR Life Sciences Corp.; Oakmont, PA) to identify and exclude mice displaying abnormal physiology.

The mouse's head was shaved to expose the scalp. Betadine was applied and a surgical drape was placed over the mouse to expose only the region of the head required for the surgical procedure. A midline sagittal incision was made from bregma to lambda to expose the skull. A 3.0 mm circular craniotomy was performed along the sagittal incision at the midpoint between bregma and lambda. Care was taken to maintain integrity of the underlying dura mater. The surgical hub was then attached to the craniotomy site using a sterile Leur-Loc syringe hub, which was cut away from a 20 gauge needle, using a cyanoacrylate adhesive. Once a complete seal between the hub and the skull was visually confirmed, a layer of dental cement was applied around the base of the hub to reinforce and maintain correct positioning of the hub. Once the dental cement had cured, the scalp surrounding the hub was sutured with 5-0 prolene suture. Topical Lidocaine was applied for numbing, and Bacitracin ointment were applied to the site to prevent bacterial infection**.** The mouse was then removed from anesthesia, placed in a cage warmed by a thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA), and monitored until fully revived at which time post-operative recovery assessment, via toe pinch, was performed. This would take approximately 60-90 minutes.

To induce a mild traumatic brain injury, the mouse was re-anesthetized as previously described using 4% isoflurane. A central fluid percussion apparatus was used to administer the injury. A spacing tube was used, in which the male end was fixed to the hub. Meanwhile, the saline filled female end of the hub-spacer assembly was attached to the male end of the fluid percussion apparatus (Custom Design & Fabrication; Virginia Commonwealth University;

Richmond, VA). A mild to moderate severity fluid percussion injury (1.7 – 1.85 atmospheres) was induced by raising and releasing a pendulum onto a fluid filled piston, causing a succinct fluid pressure pulse to impact the intact dura matter. The maximum of each pressure pulse was measured using a transducer and displayed on a storage oscilloscope (Tektronix 5111, Beaverton, OR). Post-injury, each mouse was observed until spontaneous respiration resumed. Both hub and dental cement were detached together, and the incision was immediately sutured prior to the animal regaining consciousness. Response time for the following reflexes were tested: toe pinch, tail flick, and righting. Afterwards, animals were moved to a warmed cage for post-injury observation and were closely monitored until recovery was confirmed at which time the animal was returned to the vivarium. All procedures were performed for the sham animals with the exception of releasing the pendulum toward the fluid percussion apparatus.

2.3 Number of animals per age and injury group

Aged injured animals were recovered for 1 day (n=5) or 3 days (n=8). Aged Sham animals (n=4) were recovered for 1 day or 3 days, but grouped together into aged sham group. Young injured animals were recovered for 1 day (n=4) or 3 days (n=4). Young Sham animals (n=4) were recovered for 1 day or 3 days, but were grouped together into one Young sham group (**Table 2.1**). One animal was excluded for prolonged recovery time indicated by prolonged righting response.

Table 2. 1. Number of mice per age/injury groups

Group	Aged Sham	Aged Injured 1 day recovery	Aged Injured 3 day recovery	Young Sham	Young Injured 1 day recovery	Young Injured 3 day recovery
n =	4	5	8	4	4	4

2.4 Perfusion and tissue preparation

Following the predetermined recovery periods, animals were intraperitoneally injected with 0.016ml/gram body weight of 2.5% 2'2'2' tribromoethanol (avertin) (Sigma-Aldrich, St Louis, MO) to anesthetize the animal prior to perfusion. Following appropriate anesthesia, which was confirmed using a combination of toe pinch and corneal reflex, the mouse was placed on a dissecting block and stabilized with the ventral side up. An incision through both the skin and underlying musculature was made from the abdomen to the sternum. Starting from the sternum, two subsequent incisions were made to each axilla resulting in a 'Y' shaped incision. The diaphragm was then dissected away from the ribs and pericardial sac and the anterior chest wall were removed to provide access to the heart. A 22 gauge, 1 inch needle was then carefully inserted into the left ventricle making sure that neither the interventricular septum nor the aorta was punctured.

After a 0.9% NaCl flush for a minimum of 5 minutes, or until the perfusate flowed clear, the mouse was transcardially perfused with 0.1 Millonigs buffer containing 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA). Once muscular twitching had ceased, the fixative solution was continued for 10 minutes, at a rate of 7 milliliters per minute, resulting in a mild fixation, optimized for immunohistochemical labeling.

Following the perfusion, the brain was immediately harvested and placed in phosphate buffered saline (PBS) (137 mM NaCL, 10 mM Na2HPO4, 1.8 mM KH2PO4, 2.7 KCl, with a pH of 7.4) containing 30% sucrose and maintained at 4°. After 48 hours of incubation, brains were removed from the PBS-containing sucrose solution. The olfactory bulbs were removed and the remaining brain was frozen in Optimal Cutting Temperature (OCT) (Tissue-Tek, Torrance, CA) and sectioned at 40 µm using a Leica CM 1850 cryostat. Fifteen sets of six 40 µm sections were

collected coronally. Sections were collected from 2.5 mm posterior to the bregma to 1.1 mm anterior to the bregma (Paxinos and Franklin, 2003).Fifteen sets of six sections were collected and placed on ProbeOn Plus slides (Fisher Scientific, Loughborough, UK) and stored at -80°C as previously described (Clark et al., 2016).

2.5 Immunohistochemical labelling

AnkyrinG, NeuN, APP

Excess OCT was trimmed from the edge of the sections with a single edge razor blade. Using a PAP pen (Daido Sangyo Co., Ltd. Tokyo, Japan) a hydrophobic perimeter was drawn around the tissue and left to dry. 10 mM sodium citrate solution was pre-heated to 80°C for 10 minutes. Slides were then immersed in the 10 mM sodium citrate solution and put back in the oven for an additional 30 minutes followed by three 5 minute rinses in PBS. This was followed by 15 minutes of incubation in freshly made blocking solution. The blocking solution contained 0.5% Triton X-100 and ~10% cold water fish skin gelatin in PBS. 250 µl of blocking solution were pipetted onto each slide. After 15 minutes, the blocking solution was removed by tilting the slide, and dabbing it with Kimwipes (Kimberly- Clark Worldwide, Inc., Roswell, GA). The slides were then put in PBS for three 5 minutes rinses. Antibodies directed against AnkyrinG (AnkG) (Mouse monoclonal IgG2a; 1:500), amyloid precursor protein (APP) (Rabbit polyclonal IgG; 1:1000), and NeuN (Mouse monoclonal IgG1; 1:500) were diluted in the previously described blocking solution to prepare a triple label primary antibody solution **(Table 2).** 250 µl of the primary antibody solution was added to each slide. Slides were then incubated overnight in an opaque, humidified container at 4°C.

On the following day, the slides were placed in PBS for three 5 minutes rinses. In a similar manner to the primary antibody incubation, the slides were first incubated with the blocking solution for 15 minutes, followed by three 5 minutes rinses in PBS. Afterwards, the slides were incubated in secondary antibody solution for 90 minutes at room temperature. IgG2a anti mouse conjugated with a fluorescent tag with an excitation of 568 nm (1:500), IgG anti rabbit 488 nm (1:500), and IgG1 anti mouse 647 nm (1:500) were diluted in the previously described blocking solution to prepare the secondary antibody solution **(Table 2.2).** Prior to incubation, the secondary antibodies were centrifuged for 5 minutes at room temperature at a speed of 16,000 g.

After the secondary antibody incubation, the slides were rinsed 3 times for 5 minutes each in 0.1M PBS. Afterwards, 250 µl of BisBenzimide were pipetted onto each slide and incubated for 5 minutes; this was followed by PBS rinsing as described above. Coverslips were carefully mounted on the slides using 2 drops of Vectashield™ (Vector Labs, Burlingame, CA). Slides were then sealed using nail polish, and left to dry.

2.6 Image collection using confocal microscopy

Images of AnkG and NeuN labelled sections were acquired using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) housed in the VCU Department of Anatomy and Neurobiology Microscopy Facility. Image collection was restricted to cortical layer V. Images were collected in a mid-lateral position of each hemisphere of the neocortex (**Figure 2.1**).

Table 2.2 Primary and Secondary antibody concentrations for immunohistochemistry

Table 2: Immunohistochemistry Reagents

AnkyrinG for Axonal Initial Segment

-Primary: AnkyrinG (Mouse) 1:500

-Secondary: IgG2a 568 nm (Anti-Mouse) 1:500

NeuN for Cell Bodies

-Primary: NeuN (Mouse) 1:500

-Secondary: IgG1 647 nm(Anti-Mouse) 1:500

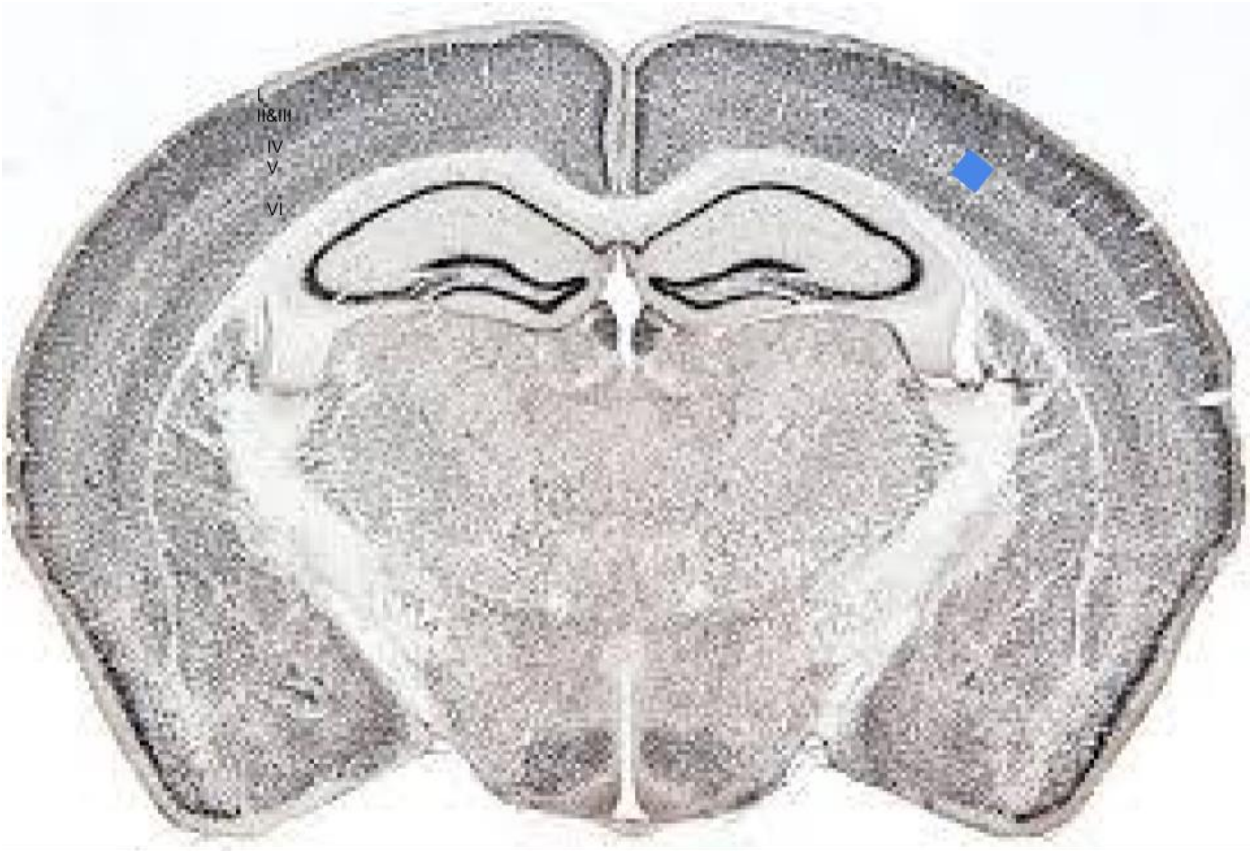
APP for Axonal Injury Swelling

-Primary: APP (Rabbit) 1:1000

-Secondary: IgG 488 nm (Anti-Rabbit) 1:500

Figure 2.1: Colored rectangular area represents the location of AIS imaging.

Image of a mouse brain section at 1.46 mm posterior to the bregma labeled with Nissl (Franklin and Paxinos, 2008). Images were taken at the mid-lateral region of the neocortex layer V represented by the blue square to quantify AIS counts, AIS length and NeuN positive cell bodies in all the mice group.



For the AnkG and NeuN labeling, maximum intensity projection images were obtained using a 30 µm z-stack. Each slide contained six sections; 2 images were collected per section resulting in twelve images acquired per animal.

For the analysis of APP swellings, which are indicative of the axonal injury, APP images were collected independently of AnkG and NeuN. Maximum intensity projection images were obtained using 20 µm range z-stacks. Three sections were imaged per slide; 3-6 images were collected depending on the size of the neocortex tiling the lateral half of the hemisphere (**Figure 2.2**). 15 images per animal; 5 images in each of the 3 sections using a 40x objective lens with a numerical aperture of 1.3. Imaging was initiated at the lateral aspect of the cortex layer V with subsequent adjacent images collected medially at previously described (Lafrenaye, 2015).

Spectral unmixing was employed to remove auto-fluorescence that resulted from lipofuscin (Clark et al., 2017), that can interfere with APP swellings quantification (**Figure 2.3**).

2.7 Image and statistical analysis

Cortical volume analysis was performed using modified Cavalieri principle (Benusa et al., 2017). Unbiased stereology was performed using every fifteenth section from the sections spanning the cortical region 1.1 mm anterior to Bregma to 2.5 mm posterior to Bregma and analyzed to estimate cortical volume of the region of interest. Each reference space was outlined with a 2X objective and analyzed using a point-grid analysis, sampling the regions of interest. Samples were counted in a blind manner and volumes calculated using an Olympus BX51 microscope (Center Valley, PA) and newCAST software (Visiophram, Hoersholm, Denmark). (n=4-7 mice per treatment group).

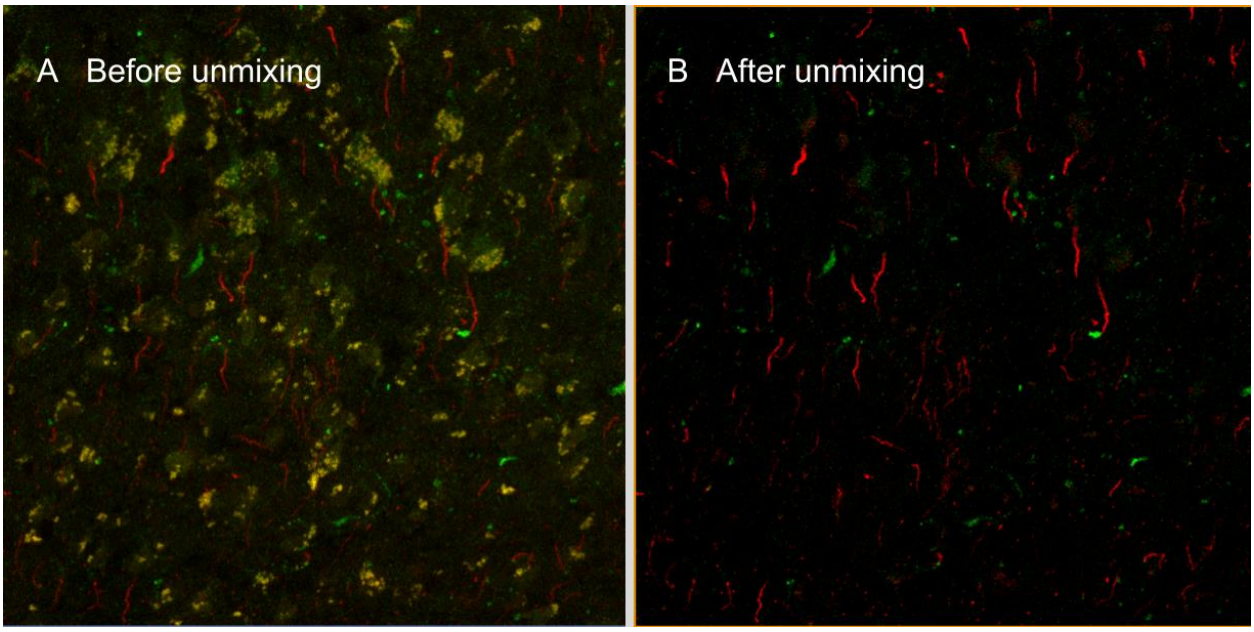
Figure 2.2: Colored area represents the location of APP imaging.

Image of a mouse brain section at 1.46 mm posterior to the bregma labeled with Nissl (Franklin and Paxinos, 2008). The blue curve represents the location of serial imaging done to survey the lateral region of layer V for APP accumulations in all mice groups.

Figure 2.3: Spectral unmixing in aged mice

Representative images taken by the confocal at layer V of aged mice brain in the midlateral region between 1.1 mm anterior to the bregma and 2.36 mm posterior to the bregma. Immunohistochemical labeling of AnkyrinG, shown in red, and APP, shown in green. Spectral unmixing was employed to remove auto-fluorescence that resulted from lipofuscin, shown in yellow in image **A.**

A) Aged mice before spectral unmixing. **B**) Aged mice after spectral unmixing.



AnkG: red
APP: green
Yellow: Lipofussin

Using ImageJ software (NIH), the axonal initial segment (AIS) length was measured by tracing the AnkyrinG label using the freehand tool (**Figure 2.4**). The measurements were collected in pixels and then converted to microns in an Excel spreadsheet. Any AnkG measurement that was less than 10 µm was excluded from the study to prevent counting nodes of Ranvier as previously described (Clark et al., 2016). To ensure no AIS were excluded, results were also calculated with the exclusion criteria set at 5 µm but the change did not yield an effect on our results. Since all AISs were traced per image, these tracing data were also used to determine the number of AISs per section. In addition to recording the number of AIS, the number of NeuN positive cell bodies was also counted using ImageJ software (**Figure 2.4**), and added to the Excel spreadsheet. The number of APP axonal swellings was analyzed manually using the particle analysis function in ImageJ software (NIH, Bethesda, MD, USA). The number of APP swellings per unit area was quantified for each image and averaged for each animal as previously described (Lafrenaye et al., 2015). In our assessment of the effect of injury and aging, there were two independent variables and more than two groups. Thus, two-way ANOVAs with Tukey's Honest Significant Difference (HSD) post hoc tests were performed for mean AIS pFOV(per field of view), NeuN pFOV, AIS/NeuN ratio, mean AIS length, APP swellings pFOV comparisons to assess the effect of injury in aged and young mice by multiple comparisons of young sham to young injured mice in 1 day and 3 days injury, and aged sham to injured mice at 1 day and 3 days recovery point. In our assessment of the effect of aging independent of injury, there were only two groups with one independent variable, aging. Thus, t-tests were performed for mean AIS pFOV, NeuN pFOV, AIS/NeuN ratio, mean AIS length, APP swellings pFOV comparisons to assess the effect of aging independent of injury by comparing aged sham mice to

young sham mice. All graphing and statistical analyses were performed using GraphPad Prism version 6.03 for Windows (GraphPad Software, San Diego, CA).

Table 2.3 Confocal Microscopy Settings

Confocal Microscope Settings

AnkG/NeuN Acquisition:-

Acquisition Mode:
Objective: 40x_cal
Scan Mode: Stack
Averaging: 2
Scan Mode: stack
Bit Depth: 16 bit
Scan Area:
Image size: 215.13 micron x 215.13 micron / pixel

Channels:-
- 2 Channels total: AnkG, NeuN
- Lasers: 561 nm (28.5%), 635 nm (20.1%)

Z-Stack:-
- Range: 30 um
- Slices: 31
- Interval: 7.56 s
- Optimal (Nyquist Sampling) for 30 microns sections

APP Swellings acquisition:-

Acquisition Mode:
Objective: 40x_cal
Scan Mode: Stack
Averaging: 1
Scan Mode: Frame
Bit Depth: 8 bit
Scan Area:
Image size: 215.13 micron x 215.13 micron / pixel

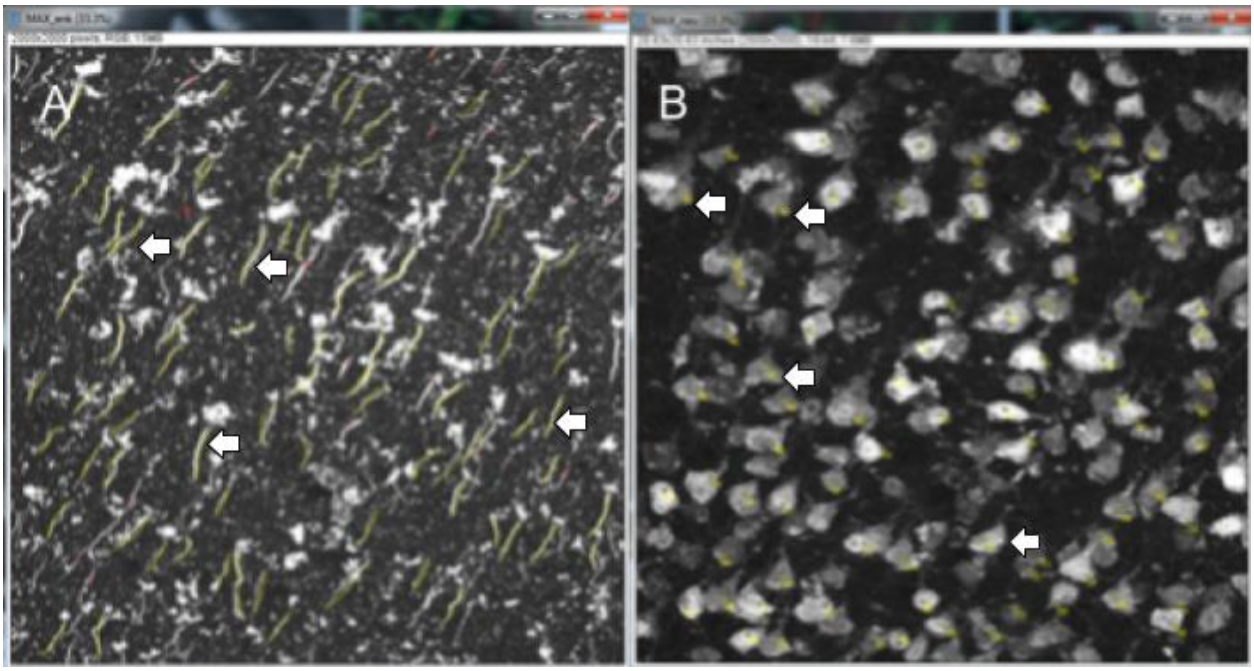
Channels:-
- 3 Channels total: APP, AnkG, Lipofussin
- Lasers: 561 nm (28.0%), 488 nm (10.0%)

Z-Stack:-
- Range: 20 um
- Slices: 25
- Interval: 7.59 s

Optimal (Nyquist Sampling) for 20 microns sections

Figure 2.4: AIS tracing and NeuN positive cell body count

Image capturing the freehand tracing property in ImageJ (NIH, Bethesda, MD, USA), the AIS was traced (A) with a yellow line indicating that it has been traced; arrows pointing to traced AIS. Using the point feature in ImageJ (NIH, Bethesda, MD, USA), NeuN count was done (B). Arrows pointing to counted NeuN positive cell bodies. Images used were taken in the midlateral region between the 1.1 mm anterior to the bregma and 2.36 posterior to the bregma.



CHAPTER 3

RESULTS

3.1 Righting reflex was significantly suppressed following cFPI in both young and aged mice

A common function test following central fluid percussion injury (cFPI) is the righting reflex**.** Loss of righting reflex has been described as a means to measure loss of consciousness because experimental data have indicated a close correlation between LORR in laboratory rodents and LOC in humans over a range of anesthetic concentrations (Grimm et al., 2015). There was a significant increase in the righting reflex times when comparing young sham (2.70 ± 0.32 minutes) to young injured (6.27 ±0.38 minutes; p=0.026) and when comparing age sham (3.72 ±.0.59 minutes) to aged injured (7.86 ± 0.59 minutes; p = 0.0006) (**Figure 3.1**). Although the average recovery time for righting reflex in the young injured was ~1.5 minutes shorter than the average time for the aged sham, this difference was not statistically significant.

3.2 No significant change in cortical volume

No differences in cortical volumes were detected among any treatment groups (Young sham, Young injured at 1 day recovery, Young injured at 3 days recovery, Aged Sham, Aged injured at 1 day recovery, Aged injured at 3 days recovery) (**figure 3.2).**

Figure 3.1 Loss of righting reflex

A significant increase in the loss of righting reflex duration was observed when comparing young injured mice to young sham mice. Similar significant increase in loss of righting reflex duration was observed in aged injured mice when compared to aged sham mice. However, no significant difference was observed between age groups.

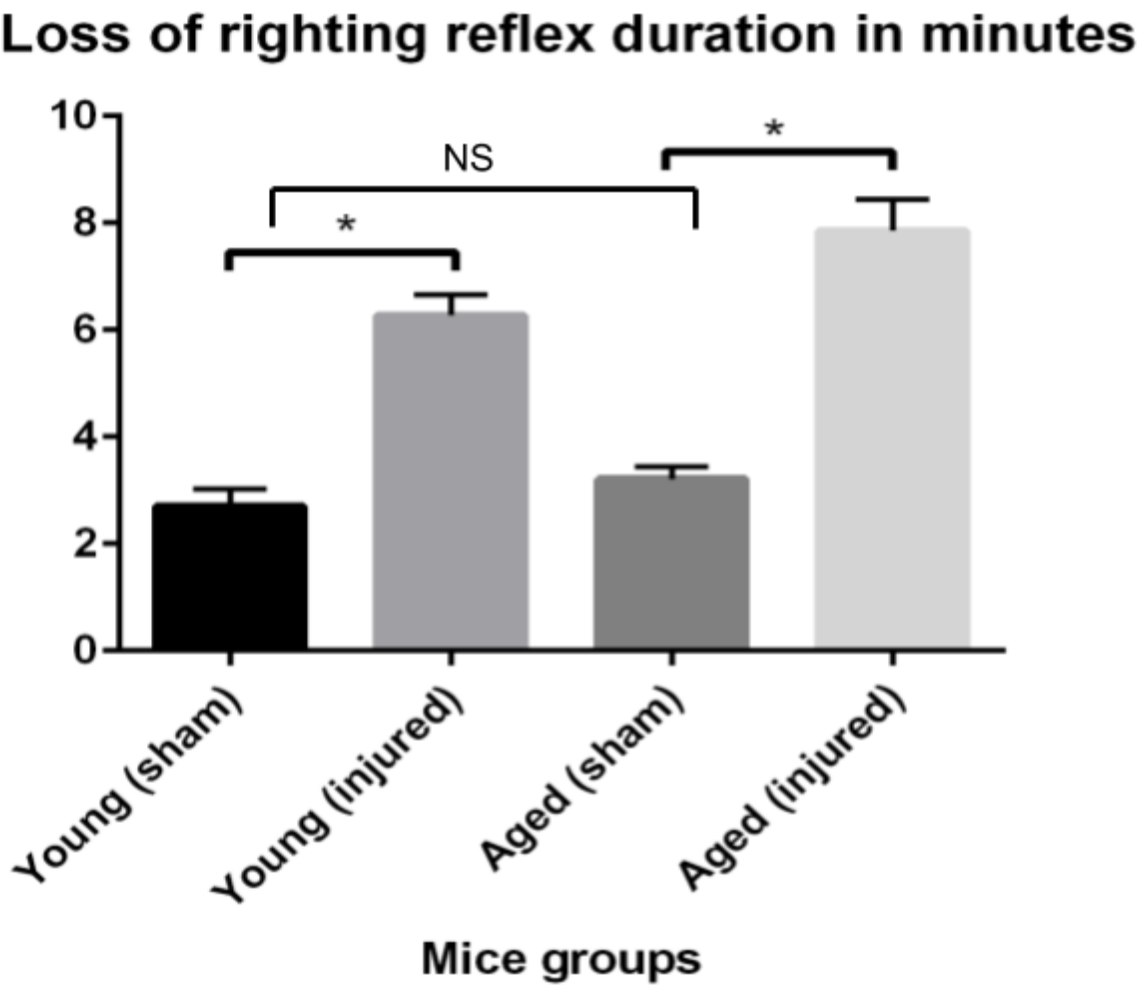
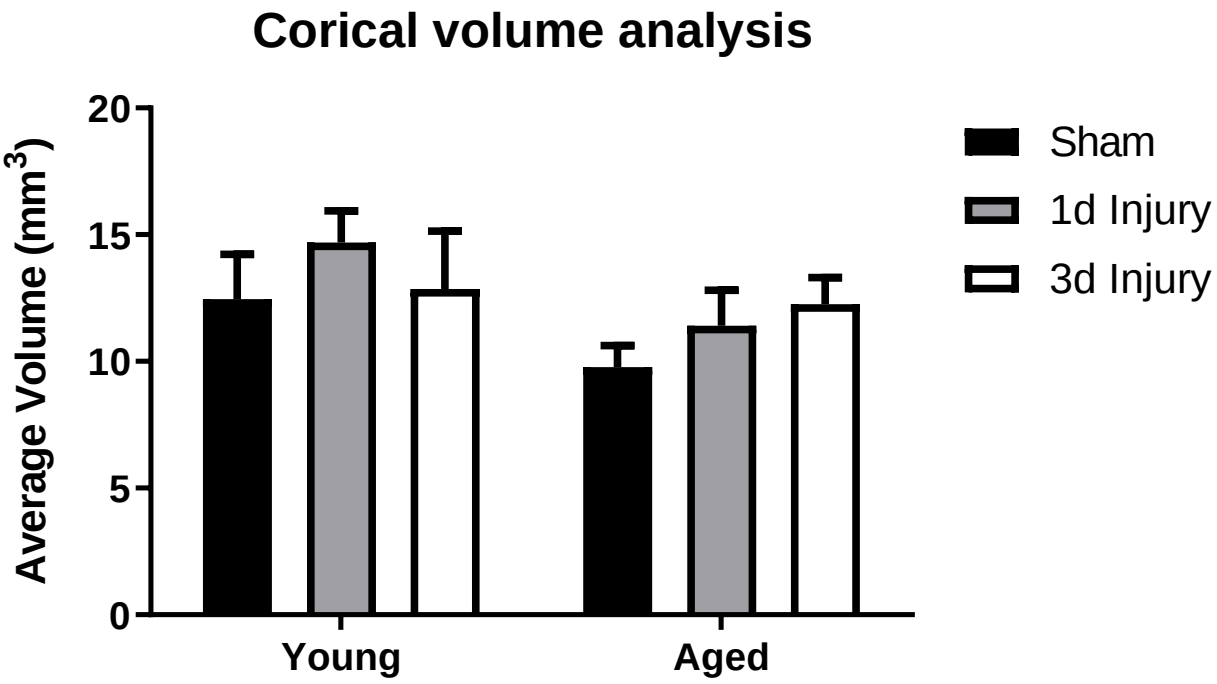


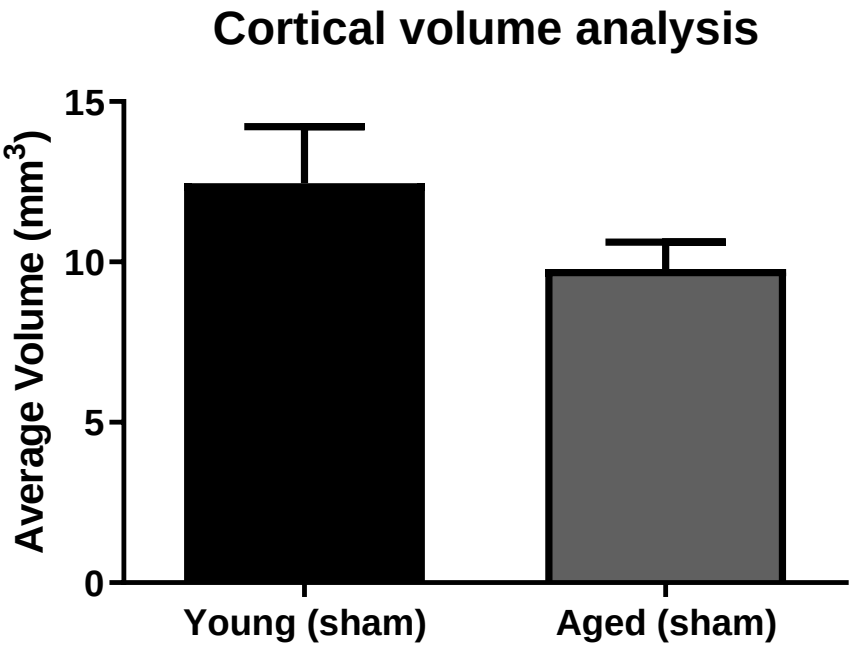
Figure 3.2 Cortical volume analysis

A. A two-way ANOVA statistical analysis of cortical volume reveals no significant change across all mice group.
B. A t-test statistical analysis reveals no significant change between young sham and aged sham.

A



B



3.3 The number of NeuN+ cells is not reduced in layer V of the cortex following mTBI

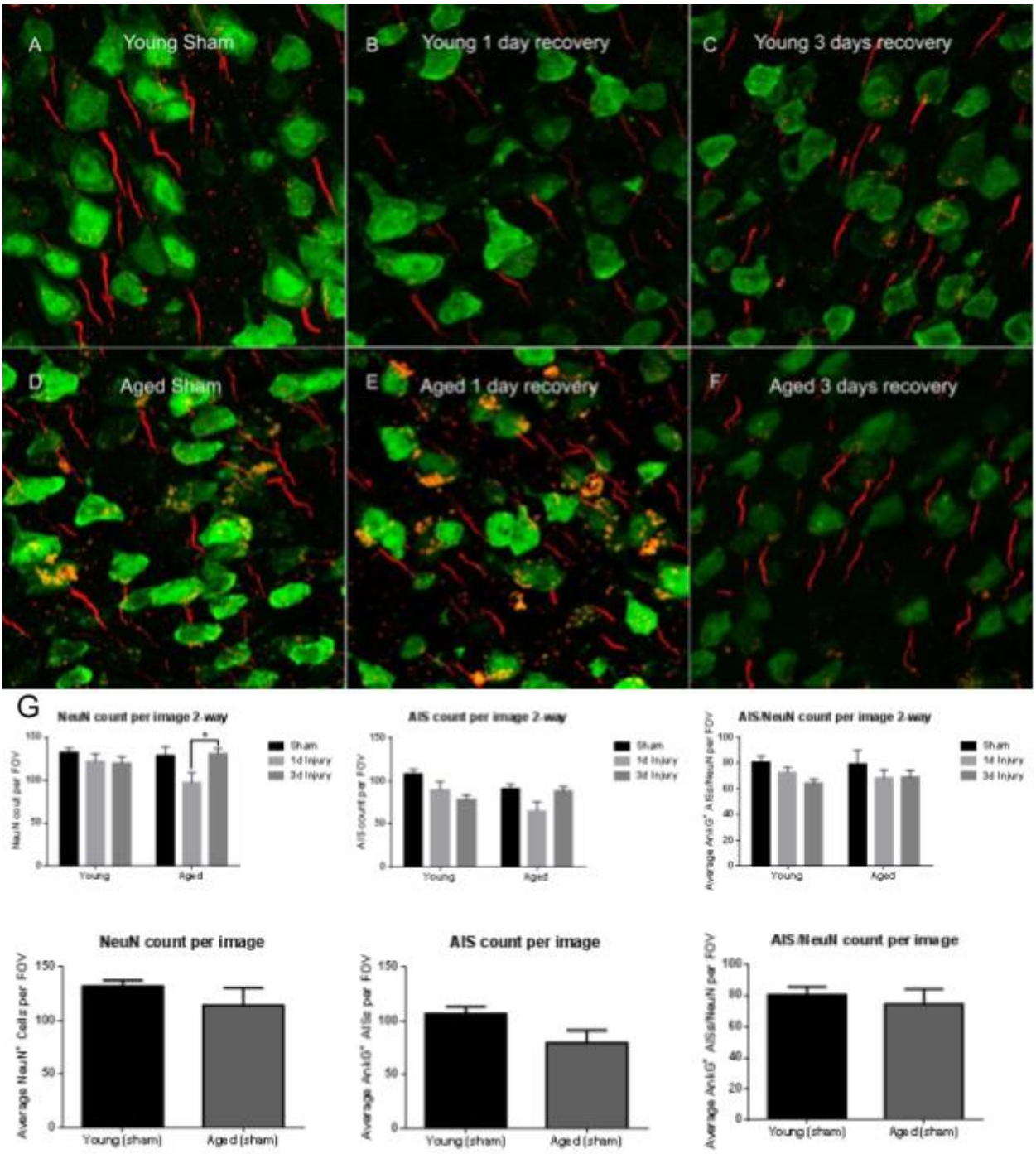
Previous studies have reported that mTBI, resulting from cFPI, does not result in neuronal cell death in layer V of the cortex (Ogino et. al, 2018). Another study that employed the blast wave model to simulate mTBI also reported no change in the number of NeuN+ cells (Baalman et al., 2013). Since these studies analyzed adult and not aged mice, we proposed that cell death may be a consequence of TBI in the aged brain. To test this hypothesis, we compared relative numbers of NeuN+ cells in layer V of the cortex among our four groups. As shown in **Figure 3.3**, in young mice our findings are consistent with these previous reports as we observed no significant change in the number of NeuN+ cells when comparing young sham mice to young injured mice at either 1 day or 3 days post injury. We also did not observe a significant change in the relative number of NeuN+ cells when comparing young sham mice to aged sham mice. There was also no reduction in NeuN+ when comparing aged sham mice to aged injured mice at 1 day or 3 days recovery. However, there was a significant increase ($p= 0.0145$) in the number of NeuN+ cells when comparing aged injured mice at the 1 day recovery point (97.36 ± 11.45) to aged injured mice the 3 days recovery point (131.03 ± 6.67).

3.4 APP accumulations show pathology due to injury and aging.

An increase in the number of APP swellings in axons is indicative of axonal damage (Blumbergs, et al., 1995; Stone et al., 2000; Greer et al., 2011). To quantitatively assess axonal damage following mTBI in the young and aged mice, we quantitatively compared the number of APP swellings in layer V of the cortex of all groups. Consistent with our hypothesis APP swellings were extremely rare in the young sham mice (0.034 APP swellings/FOV +/- 0.02)

Figure 3.3 No significant AIS/NeuN alteration with injury at young or aged mice.

Double immunolabeled, collapsed z-stack confocal images indicated no change in the percent of NeuN+ (neuronal marker) cells that exhibited an AnkG+ (AIS marker) process between young (A-C) and aged (D-F) or sham (A&D) and injured (B,C,E&F) mice. G. Qualitative assessment was confirmed by quantitative analyses.



indicating little to no axonal injury in these animals (**Figure 3.4**). As earlier studies have indicated, there was a statistically significant increase in the number of APP swellings per FOV when comparing young sham to young injured mice at the 1 day recovery point. The mean number of APP swellings in the injured young mice at the 1 day recovery time point was 3.26 +/- 0.65/FOV, which was a significant increase when compared to the young sham ($p= 0.04$). However, the number of APP swellings per FOV at the 3 day recovery time point was 2.14 +/- 0.64/FOV revealed no significant change when compared to the young sham mice.

APP swellings were also observed in aged sham mice. The mean number of APP swelling per FOV in aged mice was 1.143 +/- 0.2174, which was significantly greater than the number of APP swellings per FOV in the young sham mice (0.034 ± 0.02) ($p = 0.03$). The mean number of APP swellings per FOV in the aged injured mice was 3.46 +/- 1.02 at the 1 day recovery time point and 3.27 +/- 0.84 at the 3 days recovery time point. There was no statistically significant difference between the aged sham mice and the aged injured mice at either the 1 day or 3 days recovery time points.

3.5 AnkG labelling revealed shortening of AIS due to aging independent of injury

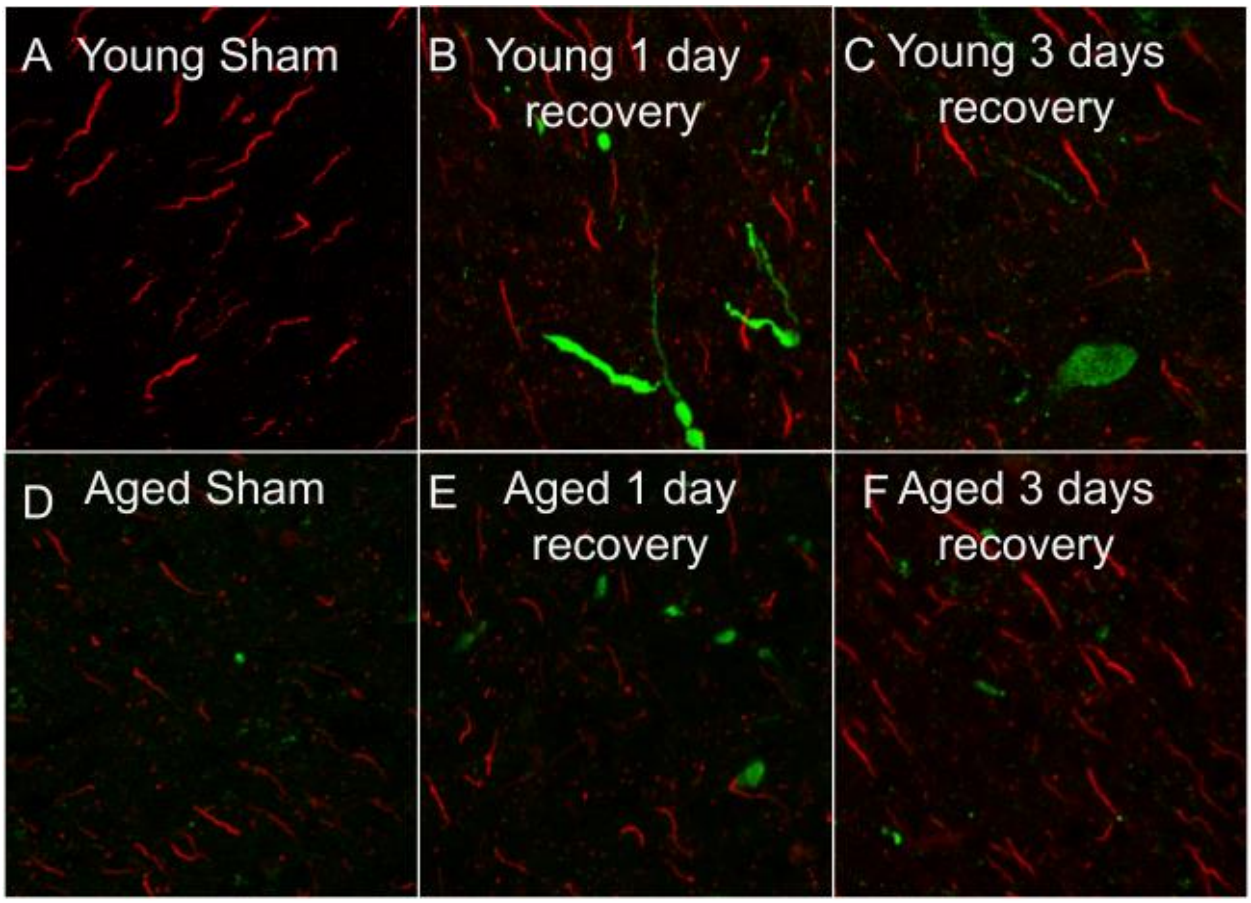
As our APP findings suggested an increase in axonal injury following mTBI, we further assessed axonal damage by analyzing structural changes of the axon initial segment (AIS). Previous work from our group has shown that the initial region of the axon, also known as the perisomatic region, is specifically prone to injury as a result of mTBI (Greer et al., 2011; Singleton et al., 2002; Vascak et al., 2017). Additionally, our laboratory reported that neuroinflammation, as assessed by microglial reactivity, results in the disruption of AISs of cortical neurons (Benusa et al., 2017; Clark et al., 2016). Since previous studies have reported significant increase in

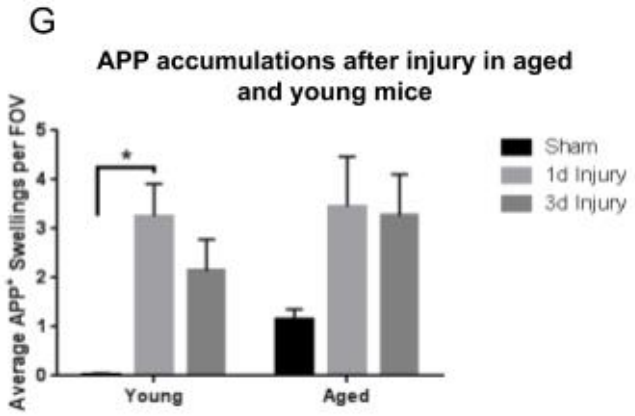
Figure 3.4 Significant change in APP swellings count per image due to injury and aging independently.

Confocal images of APP swellings in young sham mice(**A),** young injured mice at 1 day recovery (**B),** young injured mice at 3 days recovery **(C)**, aged sham mice (**D**), aged injured mice at 1 day recovery (**E**), aged injured mice at 3 days recover (**F**)

G. A Two-way ANOVA comparison indicates a significant increase, represented by "*", in the number of APP swellings in the young injured mice at the 1 day recovery point as compared to the young sham animals.

H. A t-test comparison indicates an increase in APP accumulations when comparing aged sham mice to young sham mice.





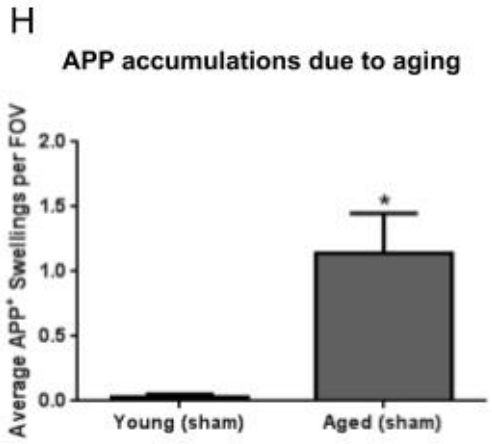


Table 3.1: Mean APP accumulations per field of view

Mice group	Young Sham	Young Injured 1 day recovery	Young Injured 3 days recovery	Aged Sham	Aged Injured 1 day recovery	Aged Injured 3 days recovery
Mean number of APP accumulations	0.035 ± 0.02	3.26 ± 0.65	2.14 ± 0.64	1.05 ± 0.18	3.46 ± 1.02	3.27 ± 0.84

neuroinflammation in the aged brain, which we have qualitatively confirmed based on microglial morphologic assessment (**Figure 1.2**), we proposed that the AIS may be vulnerable to injury. To quantitatively compare AIS structure, we analyzed AIS number and length.

No change in AIS length was observed in either the young or aged mice consequential of injury (**Figure 3.5**). Next, we compared AIS length between ages since we postulated that age-dependent inflammation (Dilger et al., 2008; Godbout et al., 2006; Johnson et al., 2006; Perry et al., 2004) would predispose the aged brain to increased injury. Interestingly, AIS length decreased by 0.86 µm from young sham to aged sham, and this reduction in length was statistically significant (p value < 0.05).

3.6 No significant change in AIS per field of view

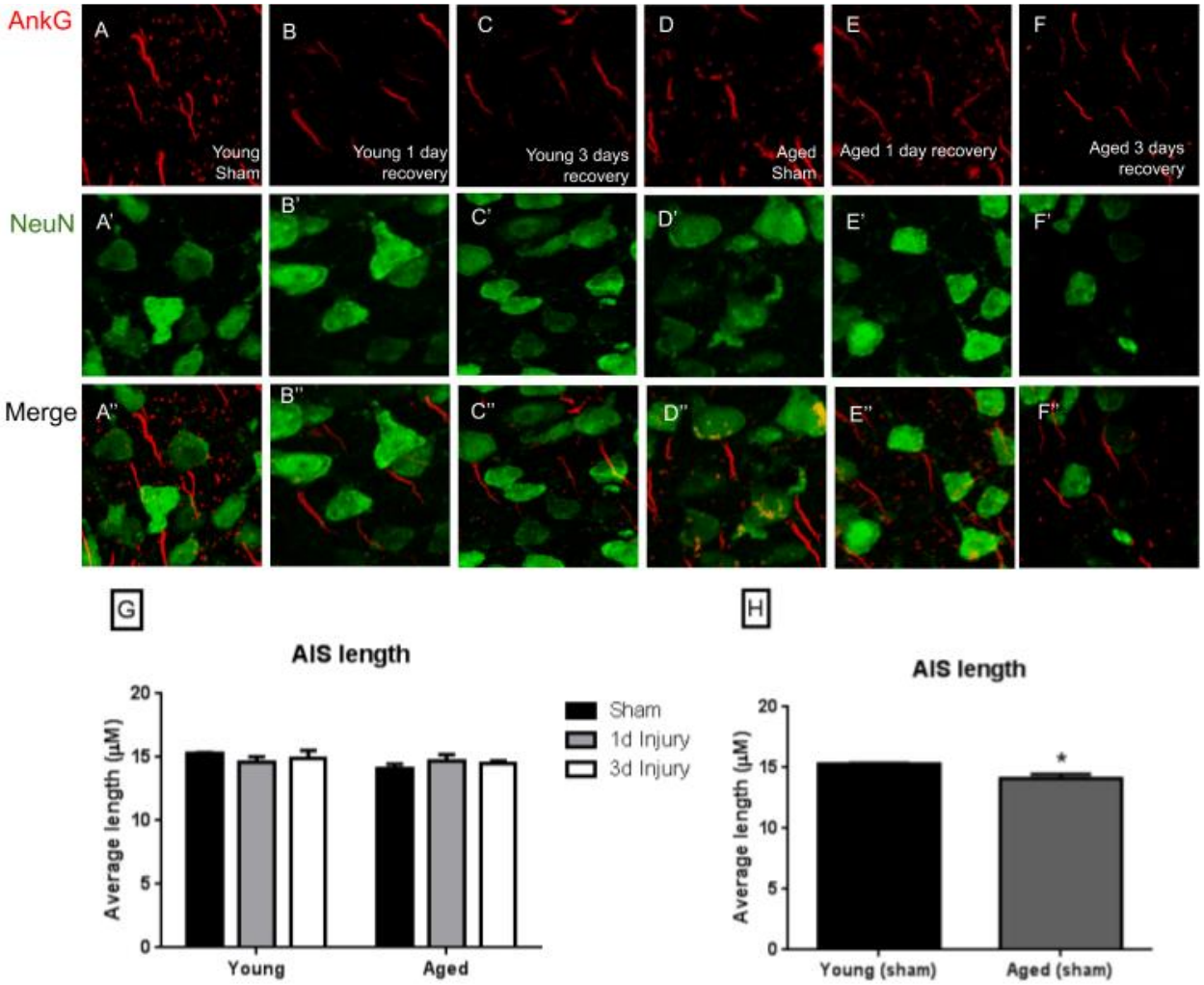
As mentioned earlier, AIS disruption, as a consequence of mTBI, has been previously reported by our group (Greer et al., 2012; Vascak et al. 2017) and others (Baalman et al., 2015). However, to our knowledge, no study has shown a reduction in the number of AISs following TBI. Although not reported following TBI, AIS number has been reported to be reduced following ischemic (Pottier et al., 2012) and inflammatory insults concomitant with increased microglial reactivity (Benusa et al., 2017; Clark et al., 2016). Since we have previously proposed that microglia reactivity drives the loss of AIS numbers and since we (**Figure 1.2**) and others (Faden et al., 2016; Simon et al., 2017) have reported increased microglial reactivity with age, we proposed that AIS numbers would be reduced in the aged brain following mTBI. Based on AIS number per field of view (FOV), we observed no significant change in the number of AnkG positive AISs when we compared the young sham mice to the young injured mice following

Table 3.2: Mean AIS length measurements

Experimental Group	**Mean length in um**
Young Sham	15.26 µm ± 0.1 µm
Young 1 day recovery	14.78 µm ± 0.2 µm
Young 3 days recovery	15.44 µm ± 0.3 µm
Aged Sham	14.40 µm ± 0.2 µm
Aged 1 day recovery	14.82 µm ± 0.3 µm
Aged 3 days recovery	14.33 µm ± 0.1 µm

Figure 3.5 Reduced AIS Length with Age

Using single immunolabeled, collapsed Z-stack confocal images, AIS length was quantified by tracing ankyrinG (AnkG) label. All measurements of AnkG labeling less than 10 µm were excluded from analysis to ensure that nodes of Ranvier were not mistakenly assessed. To ensure no AIS were excluded, results were also calculated with the exclusion criteria set at 5 µm but the change did not yield an effect on our results. Representative images are presented in **Figure 3.5 A-F**. AIS lengths (mean ±SEM) two-way ANOVA comparing all mice groups presented in Panel **G**. AIS lengths (mean ±SEM) t-test comparing young sham mice to aged sham mice presented in Panel **H**. Although the actual reductions were only ~1µm, the AISs were significantly shorter in the aged sham mice compared to the young sham mice. Interestingly, injury, regardless of the age group, did not result in further shortening of the AISs of cortical layer V neurons. ($p < 0.05$ and indicated by *****)



either 1 day or 3 days of recovery **(Figure 3.3)**. Similarly, there was no significant change in AnkG positive AIS when comparing the aged sham mice and the aged injured mice following either 1 day or 3 days of recovery.

3.7 No significant change in AIS/NeuN ratio following injury in aged or young mice

To ensure that our analysis of AnkG positive AIS counts was not skewed by differences in neuronal density, AIS/NeuN ratio was employed. Similar to the findings for the number of AISs results, there was no significant change when comparing aged sham mice to the young sham mice following correction for neuronal density. There was no change in the AIS/NeuN when comparing young sham mice to young injured mice in either the 1 day recovery point or the 3 days recovery point. Also, there was no change in the AIS/NeuN when comparing aged sham mice to aged injured mice at either the 1 day or the 3 day recovery time point **(Figure 3.3)**.

3.8 Results summary

In summary, we observed a significant increase in loss of righting reflex (LORR) duration when comparing the injured group to the sham group in both young and aged mice. While there was a significant increase in the number of APP accumulations due to injury in young mice at the 1 day point recovery, there was no significant change in APP accumulations in aged mice due to injury at the 1 day or 3 days point recovery. However, there was a significant increase in APP accumulations when comparing young sham to aged sham mice indicating an effect of aging independent of injury. While we did not observe a change in the AIS/NeuN ratio among any of the groups due to injury or aging, we observed a significant decrease in AIS length due to aging independent of injury.

Table 3.3 : Statistical comparisons of results

Statistical values presented in the table are p, t and F. Under two-way ANOVA, F values for age, injury and interaction are generated. F(i) is the F value for injury factor. F(a) is the F value for age factor. F(int) is the F value for interaction factor.

	AIS per image	NeuN	AIS/NeuN	AIS length	APP	LORR
Young sham v young injure 1 day (two-way ANOVA)	p=0.40 t=1.47 F(i)=3.7 F(a)=2.49 F(int)=2.74	p=0.72 t=0.77 F(i)=2.79 F(a)=0.61 F(int)=2.08	p=0.71 t=0.79 F(i)=1.98 F(a)=0.01 F(int)=0.27	p=0.58 t=1.18 F(i)=0.01 F(a)=2.08 F(int)=1.26	p=0.04 t=2.61 F(i)=5.73 F(a)=1.46 F(int)=0.21	p=0.007 t=3.22 F(i)=32.15 F(a)=2.08 F(int)=0.56
Young sham v young injured 3 day (two-way ANOVA)	p=0.07 t=2.43 F(i)=3.7 F(a)=2.49 F(int)=2.74	p=0.63 t=0.92 F(i)=2.79 F(a)=0.61 F(int)=2.08	p=0.26 t=1.62 F(i)=1.98 F(a)=0.01 F(int)=0.27	p=0.87 t=0.70 F(i)=0.01 F(a)=2.08 F(int)=1.26	p=0.23 t=1.7 F(i)=5.73 F(a)=1.46 F(int)=0.21	p=0.007 t=3.22 F(i)=32.15 F(a)=2.08 F(int)=0.56
Aged sham v aged injured 1 day (two-way ANOVA)	p=0.09 t=2.27 F(i)=3.7 F(a)=2.49 F(int)=2.74	p=0.06 t=2.42 F(i)=2.79 F(a)=0.61 F(int)=2.08	p=0.51 t=1.12 F(i)=1.98 F(a)=0.01 F(int)=0.27	p=0.67 t=1.04 F(i)=0.01 F(a)=2.08 F(int)=1.26	p=0.14 t=1.97 F(i)=5.73 F(a)=1.46 F(int)=0.21	p<0.0001 t=4.97 F(i)=32.15 F(a)=2.08 F(int)=0.56
Aged sham v aged injured 3 days (two-way ANOVA)	p=0.99 t=0.3 F(i)=3.7 F(a)=2.49 F(int)=2.74	p=0.98 t=0.20 F(i)=2.79 F(a)=0.61 F(int)=2.08	p=0.50 t=1.13 F(i)=1.98 F(a)=0.01 F(int)=0.27	p=0.88 t=0.67 F(i)=0.01 F(a)=2.08 F(int)=1.26	p=0.14 t=1.98 F(i)=5.73 F(a)=1.46 F(int)=0.21	p<0.0001 t=4.97 F(i)=32.15 F(a)=2.08 F(int)=0.56
Young sham v aged sham (t-test)	p=0.10 t=1.93 F=1.72	p=0.76 t=0.32 F=3.76	p=0.89 t=0.14 F=4.89	p=0.02 t=3.12 F=20.82	p=0.0023 t=5.08 F=118.5	p=0.24 t=1.32 F=1.46

Chapter Four

Discussion

4.1 Synopsis

The focus of this study was to determine the effect of diffuse traumatic brain injury and aging on structural alterations of the AIS, and whether these two variables interact to exacerbate injury. Previous observations from our laboratory, combined with published reports (Ritzel et al., 2019; 2018; Sharaf et al., 2013; Sun et al., 2013; Zoller et al., 2018) indicated that microglia in the aged brain are reactive and presumably primed in an M1 (pro-inflammatory) state of activity. Since our previous work implicates reactive microglia in driving axonal pathology (Clark et al., 2016; Benusa et al., 2017), we proposed that axonal burden would be significantly increased in the aged brain following TBI.

To initially assess axonal injury, we quantitatively compared the number of APP swellings in young adult and aged brains, with and without injury. The extent of axonal injury was significantly increased in the young injured compared to the young sham mice, which has been consistently reported (Singleton et al., 2002; Kelley et al., 2006, 2007; Greer et al., 2011; Vascak et al., 2017); however, surprisingly, this increase in injury was not observed in the aged injured compared to the aged sham animals. To further assess axonal injury, we specifically assessed structural changes of the axon initial segment (AIS). We focused on the AIS since our laboratory has reported that the AIS is a target of attack by reactive microglia and their inflammatory products (Clark et al., 2016; 2017; Benusa et al., 2017). Moreover, previous work has shown that the AIS is specifically targeted following TBI (Greer et al., 2013). To our surprise, we observed no consistent, significant disruption in the AIS with regard to number or

length in either young or aged brains following TBI (Table 3.2). Taken together, our findings indicate that age does not significantly increase axonal burden, at least with regard to structural alterations within the AIS, following mild TBI. However, we did observe a significant decrease in AIS length due to aging independent of injury accompanied by an increase of APP accumulations.

4.2 Loss of righting reflex in aged and young mice

Consistent with previous reports of loss of righting reflex duration in mTBI mice (Greer at al., 2011; Hanell et al., 2015), both aged mice and young mice exhibit an increase in the length of time required for re-establishment of the righting reflex following mTBI (**Figure 3.1**). On the other hand, there was no significant change in the righting reflex due to aging independent of injury. We did not observe a significant difference between the righting reflex duration between the young injured mice and aged injured mice. Hence, mTBI resulted in similar LORR in both aged and young mice.

Loss of righting reflex has been described as a means to measure loss of consciousness because experimental data have indicated a close correlation between LORR in laboratory rodents and LOC in humans over a range of anesthetic concentrations (Grimm et al., 2015). In cFPI, the mechanical injury generates neurological signs of transient behavioral suppression that has been described to resemble signs of unconsciousness in humans (Vascak et al., 2017). Based on our observed results with regard to loss of righting reflex, both aged and young mice experience a significant increase in LORR after injury indicating that they have experienced the same level of mTBI (Greer at al., 2011; Hanell et al., 2015).

4.3 APP swellings indicate axonal injury due to injury and aging independently

Previous studies have used APP swellings as an indicator of axonal injury (Singleton et al., 2002; Kelley et al., 2006, 2007; Greer et al., 2011; Vascak et al., 2017). In addition to axonal injury, in general, APP-labelled swellings, which have long been associated with morbidity following TBI in humans and animal models (Ganenareli et. al, 1982; Povlishock et al., 1995; Scheid et. al, 2006; Browne et. al, 2011; Johnson et al., 2012), have been used as the classic marker of DAI (Singleton et al., 2002; Kelley et al., 2006, 2007; Greer et al., 2011; Vascak et al., 2017). Consistent with these previous studies, we observed a significant increase in the number of APP swellings in young injured mice when compared to young sham mice. Similar to our observations in regards to LORR, there was no significant change in the number of APP swellings when comparing young injured mice to aged injured mice. Previous studies have reported that the number of APP axonal swellings decrease from 1 day to 3 days post injury (Greer et al., 2011; Singleton et al., 2002). However, in our study, there was no change in the mean number of APP swellings per field of view from 1 day to 3 days post injury in either aged or young mice. While APP transcription upregulation has been reported in TBI patients, its axonal transport is interrupted due to diffuse axonal injury; this results in deposition of APP and its products in axonal "bulbs" (Hayashi et al., 2015).

As mentioned earlier, DAI has been identified by the presence of APP+ swellings in the proximal region of axons; these APP swellings are indicative of impaired protein transport in the proximal axonal segment remaining attached to the neuronal soma following disconnection (Wang et al., 2011; Hånell et al., 2014). The failure of the axonal transport system causes a gradual accumulation of both transported vesicles and organelles, which progresses to axonal

swelling and eventually axotomy (Buki et al., 2006; Hånell et al., 2015). It has also been reported that the APP immunoreactivity has been restricted to the perisomatic/proximal axonal swelling (Greer et al., 2013; Vascak et al., 2018). In contrast, the disconnected distal axonal segments have been reported devoid of APP immunoreactivity (Greer et al., 2011; Wang et al., 2011; Hånell et al., 2015; Vascak et al., 2018).

Based on our results, we report that TBI independent of aging results in impaired axonal transport. Impaired axonal transport was observed due to aging independent of injury. However, there was no exacerbation of the impaired axonal transport when aging and injury were combined.

4.4 No significant change in effect of cFPI on neuronal cell death in aged and young mice.

Previous studies have reported neuron reduction in the cortex and hippocampus within 3 days following mTBI as assessed with NeuN immunolabeling (Rachmany et al., 2013; Tashlykov et al., 2007 & 2009). This apparent neuronal cell death has been attributed to P53 - dependent neuronal cell death (Rachmany et al., 2013; Muir et al., 1999; Plensila et al., 2007; Schober et al., 2009). Specifically in the cortex, a previous study reported limited cell death characterized by a reduction of NeuN+ cell bodies density (Gao et al., 2011). However, our study reports no change in NeuN+ cell body density when comparing sham mice to injured mice at both 1 day and 3 days regardless of age. Hence, based on our observations of NeuN+ cell bodies, aging does not affect mouse susceptibility to neuronal cell death after mTBI within the 3 day window studied. Consistent with human mTBI, cFPI reproducibly evoked DAI without mass lesions, cortical contusion, or cell death (Mittl et al., 1994; Saatman et al., 2008; Andriessen et

al., 2010; Bigler and Maxwell, 2012; Yuh et al., 2013; Shultz et al., 2016). More specific to our study, in layer V, after cFPI, scattered DAI within layer V is observed, but no cell death has been reported (Singleton et al., 2002; Greer et al., 2011 and 2012). However, mTBI has been reported to induce DAI primarily within the AIS and para-AIS regions of the axons of pyramidal neurons of layer V (Greer et al., 2013).Thus, the AIS was our next target for analysis.

4.5 AIS alteration after mTBI in young mice: compensatory or disruptive?

Multiple studies have reported remodeling of AIS following ischemic injury (Schafer et al., 2009; Hinman et al., 2013) and blast wave (Baalman et al., 2013). Vascak et al. (2017) reported a decrease in AIS length in non-axotomized axons following cFPI. Here, we report no change in AIS length or count between sham and injured mice in both aged and young mice. This deviates from a previous report indicating loss of AnkG within 2 days after cFPI (Vascak et al., 2017). While Vascak et al. (2017) reported that mTBI results in a decrease in AnkG at 2 days post-mTBI within the intact axons in layer V (Vascak et al., 2017), Greer et al. (2013) report that despite traumatic axonal injury, AnkG immunoreactivity persisted within the axonal cylinder. More importantly, Vascak et al. (2017) concluded that this subtle decrease in AIS length in the intact axon attenuates AP acceleration. This hints to a compensatory mechanism to the increase in AP amplitude and decrease in after-hyperpolarization duration, AHD, in the axotomized neurons described by Greer et al. (2012). Another study reported shortening of the AnkG+ AIS in rats exposed to mTBI when compared to the control group 2 weeks post injury (Baalman et al., 2013). Baalman et al. (2013) also reported that the injured rats experienced impaired cognitive function. Several studies have shown AIS disruption association with multiple diseases

that involve an imbalance of network function (Kaphan et al., 2011; Hinman et al., 2013; Hamada and Kole, 2015; Clark et al., 2016; Benusa et al., 2017).

In light of the previously mentioned association of AIS disruption to an imbalance of network function, we employed different measures to assess AIS disruption. Our study shows no change in AIS length due to injury in both young and aged mice. To the best of our knowledge this is the first study to report the effect on mTBI on the AIS length in aged mice. Our findings in the young mice deviate from previous studies that report global, but modest, decreases in AIS length (Baalman et al., 2013). Baalman et al. (2013) employed a blast wave model to induce mTBI, while we used cFPI. Our findings also differ from the observations by Vascak et al. (2017), who report a shortening of the AISs. However, it is important to realize that in the work by Vascak et al., only intact axons were analyzed, whereas in contrast, we analyzed AIS both axotomized and intact axons.

There was no change in AIS/NeuN ratio after injury in the young mice. This was also the case in the aged mice However, TBI results in higher morbidity and mortality in the aged population (Ramanathan et al., 2012; McIntyre et al., 2013; Dams-O'Connor et al., 2013; Coronado et al., 2005). Hence, we expected to see an exaggerated disruption of the AIS after TBI in aged mice. Still, this was not the case. In addition to our analysis of the effect of mTBI on the AIS in aged and young mice, we also looked at the effect of aging on the AIS independent of injury.

4.6 AIS length decrease due to aging independent of injury

To the best of our knowledge, we are the first to report a significant decrease in AIS length in layer V pyramidal cells as a consequence of age. Using western blot analysis, Kneyshberg and Kanaan (2017) reported no difference in AnkyrinG levels in aged rats. This study also reported no change in the AIS length based on immunohistochemical analysis of AnkG in the hippocampus. Since AnkyrinG is also expressed in nodes of Ranvier, the levels that these authors reported were not limited to changes within the AIS; and since the immunohistochemical analysis was conducted in the hippocampus, our reports do not necessarily present opposing results (Kneyshberg and Kanaan, 2017). On the other hand, Ataour and Rosa (2017), using immunohistochemical approaches combined with confocal microscopy, analyzed AIS length in the aged cortex by quantifying length of the AISs of pyramidal cells in the cortical layers 2 and 3 in aged marmoset monkeys and reported that the AIS was significantly shortened by 24%. Our study reports that the AIS in layer V is shortened by 6%.

AIS shortening is likely to represent a compensatory response to changes in the excitation-inhibition balance, associated with the loss of GABAergic interneurons in the aged cortex (Atapour and Rosa et al., 2017). In our study, we did observe shortening of the AIS in the aged sham mice (**Figure 3.5**), thus this compensatory response may still be intact in the sham mice. While we observed shortening of the AIS due to aging independent of injury, there was no significant change in the AIS/NeuN ratio due to aging independent of injury. Also, previous qualitative analysis in our lab has shown that microglia in the aged sham mice to exhibit a reactive morphology (**Figure 1.2**).

4.7 Is AIS regulation in the aged brain a possible homeostatic plasticity?

Similar to our observations of shortening AIS due to aging independent of injury, Clark et al. (2016) reported shortening of the AIS with no decrease in the AIS count during the early stage of the chronic model of experimental autoimmune encephalomyelitis (EAE). Concomitant with this shortening, Clark et al. (2016) also reported that reactive microglia contact the AIS coincidental with a 75% loss of AIS in the late disease stage. In another study exploring the effect of reactive microglia on AIS integrity, Benusa et al. (2017) injected lipopolysaccharide, a known activator of microglia, and reported both a shortening of the AIS and a decrease in the number of cortical neurons that presented with intact AISs, as assessed by AnkG immunolabeling. Since both the work of Clark et al. (2016) and Benusa et al. (2017) are consistent with the possibility that reactive microglia are sufficient to drive AIS structural changes and since microglia have been reported to be reactive in the aged brain, we proposed that the age dependent shortening of the AIS that we observe in our current work may be consequential to age dependent microglia activation. Since reactive microglia can present with distinct molecular profiles, it will be of great interest to determine if the pro-inflammatory state induced by EAE and LPS is mimicked in the aged brain.

Under neuroinflammatory conditions, the microglia play a role in altering the AIS structural integrity whether by changing length or count (Clark et al., 2016; Benusa et al., 2016). With aging the microglia are primed and biased toward an exaggerated pro-inflammatory immune response to an insult, i.e. TBI. This exaggerated response is absent in the absence of an insult, (e.g. in sham mice) (Barrientos et al., 2015; Frank et al., 2010). Interestingly, the basal homeostatic proliferation rates in microglia in the aged mice have been reported to be inhibited

or impaired after TBI (Ritzel et al., 2019). For instance, microglia phagocytic activity, which is responsible for clearance and removal of damaged cells and debris, has been reported as significantly impaired in aged mice after TBI when compared to the young mice after TBI (Ritzel et al., 2019).

Aging has been reported to decrease neuronal plasticity (Ma et al., 2012; Jellinger and Attems, 2013) while aged rodents exhibit a deficiency in hippocampal neurogenesis and long term potentiation (Maher et al., 2005; 2006; van Praag et al., 2005). It has been suggested that neuroinflammation plays a role in altering neuroplasticity (Kohman et al., 2012). Aged microglia have been reported to contribute to the impaired neuroplasticity in long term potentiation via release of inflammatory cytokines (Lynch, 2010; Kelly et al., 2013). Aging induces a shift in microglia phenotype toward more proinflammatory than anti-inflammatory profiles resulting in priming and sensitizing the microglia (Patterson, 2015; Norden and Godbout, 2013).

It has been reported that with aging, microglia is impaired in restoring homeostasis after TBI (Ritzel et al., 2019). Aged microglia have been reported to have impaired neuroplasticity in long term potentiation due to release of inflammatory cytokines (Lynch, 2010; Kelly et al., 2013). Previous reports from our lab suggest an involvement of reactive microglia in reducing AIS length and counts in the EAE and LPS neuroinflammatory models (Clark et al., 2016; Benusa et al., 2017). This study reports a reduction in the mean AIS length with aging independent of injury. This AIS shortening may represent a compensatory response to changes in the excitation-inhibition balance, associated with the loss of GABAergic interneurons in the aged cortex (Atapour and Rosa et al., 2017). This suggests that despite the previously mentioned microglia impairment in aged mice, microglia regulation of AIS length to maintain excitation-inhibition balance is intact in aged mice as long as no additional insult is present.

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Vita

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