2014

Structural Alterations to the Axon Initial Segment Following Diffuse Axonal Injury as a Consequence of Age

William Behl
Virginia Commonwealth University

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STRUCTURAL ALTERATIONS TO THE AXON INITIAL SEGMENT FOLLOWING DIFFUSE AXONAL INJURY AS A CONSEQUENCE OF AGE

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

by

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April, 2014
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<td>AIS</td>
<td>axon initial segment</td>
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<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>ankG</td>
<td>ankyrinG</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APP</td>
<td>β-amyloid precursor protein</td>
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<tr>
<td>ATF-3</td>
<td>activating transcription factor 3</td>
</tr>
<tr>
<td>atm</td>
<td>atmospheres</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>Ca^{2+}</td>
<td>calcium ion</td>
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<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
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<tr>
<td>caspr</td>
<td>contactin-associated protein</td>
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<tr>
<td>cFPI</td>
<td>central fluid percussion injury</td>
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<td>CMSP</td>
<td>calpain-mediated spectrin proteolysis</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAI</td>
<td>diffuse axonal injury</td>
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<tr>
<td>DTI</td>
<td>diffusion tensor imaging</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>K_v</td>
<td>voltage-gated potassium channel</td>
</tr>
<tr>
<td>LOC</td>
<td>loss of consciousness</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopy</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
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<tr>
<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>Na^+</td>
<td>sodium ion</td>
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<tr>
<td>Na_v</td>
<td>voltage-gated sodium channel</td>
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<tr>
<td>NF-155</td>
<td>neurofascin-155</td>
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<td>NF-186</td>
<td>neurofascin-186</td>
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<tr>
<td>NrCAM</td>
<td>neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature™</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RTA</td>
<td>road traffic accident</td>
</tr>
<tr>
<td>SCWM</td>
<td>subcortical white matter</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
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<tr>
<td>SERCA</td>
<td>SER Ca$^{2+}$-ATPase</td>
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<tr>
<td>TAI</td>
<td>traumatic axonal injury</td>
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<td>TBI</td>
<td>traumatic brain injury</td>
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<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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<td>µm</td>
<td>micrometer</td>
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ABSTRACT

STRUCTURAL ALTERATIONS TO THE AXON INITIAL SEGMENT FOLLOWING DIFFUSE AXONAL INJURY AS A CONSEQUENCE OF AGE

By William C. Behl

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

Virginia Commonwealth University, 2014

Director: Jeffrey L. Dupree, Ph.D.
Associate Professor of Anatomy and Neurobiology

An epidemiological shift towards the elderly population has occurred in traumatic brain injury (TBI). Age is believed to be one of the strongest prognostic indicators following TBI. Diffuse axonal injury (DAI), a prevalent feature of TBI, is believed to be the primary cause for much of the morbidity and mortality associated with TBI. The pathobiology associated with DAI is believed to occur in response to the primary injury in a progressive, secondary fashion. Though the injury mechanisms behind DAI have been shown to occur at numerous sites along the axon, recent work suggests that the axon initial segment (AIS) may show specific vulnerability to DAI and be the primary site of axonal pathobiogenesis. Despite its established predilection for injury, the mechanisms responsible for the pathobiology remain largely unclear – particularly with regard to the age. The current study aims to shed light on the mechanisms responsible for injury by investigating structural alterations to the AIS following DAI in young and old mice. To address this question we have used a central fluid percussion injury (cFPI) model to induce mild DAI on 22-month old aged mice and 3-month old young mice at 3-hours and 24-hours survival time. Double-labeling fluorescent immunohistochemistry was used to
demonstrate colocalization of ankG, an AIS domain marker, and APP, a marker used to establish traumatic axonal injury (TAI). Qualitative-quantitative observations based on confocal microscopy demonstrated an increase in APP accumulation associated with AIS over time, post-injury. Initial segments displaying APP association consistently showed a significant overall shortening in young and aged groups at both survival times. No significant difference in AIS length was detected between AIS populations of young and aged mice. Qualitative findings, however, suggest that AIS degradation could be more profound with age, which could have implications on neuronal outcome.
INTRODUCTION

Traumatic Brain Injury

A Changing Epidemiology

Traumatic brain injury (TBI) is a prominent public health problem both domestically and abroad, with approximately 2 million cases diagnosed annually in the United States and over 10 million people treated each year worldwide (Langlois, et al., 2006). Each year in the United States, approximately 70,000 people die from head injuries and TBI has been labeled as a contributing factor to nearly a third (30.5%) of all injury related deaths (Faul et al., 2010). There are between 2.5 and 6.5 million Americans alive today who have suffered a TBI and survivors are often left with significant cognitive, behavioral, and communication disabilities, with some patients developing long-term medical complications, such as epilepsy (National Institutes of Health, 2002).

TBI can affect anyone, however, epidemiological risk factors such as age and gender do exist. Age-related increased risk has been associated with young children (ages 0-4), older adolescents (ages 15-19), and more senior individuals (over 65 years of age) (Faul et al., 2010). Males between the ages of 20 and 24 years old are 4 times more likely to sustain a TBI than females of similar age (Coronado et al., 2011). Half of all TBIs are due to road traffic accidents (RTA); approximately 20% are due to violence; and roughly 3% are due to sports injuries (National Institutes of Health, 2002). However, the epidemiology of TBI is changing in several Western countries, with an increasing
proportion of elderly patients admitted to the intensive care unit (ICU) (Stocchetti et al., 2012). One reason is that successful RTA prevention is leading to fewer accidents and less TBI among young people (World Health Organization, 2010). However, increased life spans and a larger elderly population also play a role in driving the TBI epidemiology shift with the majority of cases involving falls at home (Stocchetti et al., 2012).

The Aging Brain

Injury aside, the process of aging is a gradual and multiplex process, involving cells, tissue, and organs affecting the organism as whole. In the brain, the process of deterioration and progressive degradation is irreversible, implying pathological conditions that permanently affect the individual’s quality of life (Ortuno-Sahagun et al., 2014). Numerous detrimental cellular processes are known to occur, including the accumulation of reactive oxygen species, neuro-inflammation, axonal transportation pathologies, and overall neuronal atrophy.

Oxidative stress and the intrinsic role of reactive oxygen species is a key aspect to the pathobiology of normal aging (Ortuno-Sahagun et al., 2014). It is well recognized that reactive oxygen species are produced and tightly regulated to help ensure normal cellular homeostasis (Grune et al., 2012). During the normal aging process, these reactive oxygen species are known to accumulate, eventually exceeding the cell’s antioxidant capacity, causing severe damage in the form of oxidative stress (Grant et al., 2009). The production of free radicals and peroxides during oxidative stress has been linked to damage of cellular proteins, lipids, and DNA, leading to a decline in normal
physiologic function and in increased susceptibility to disease and death (Patel and Chu, 2011).

Another hallmark of the aging brain is neuroinflammation. The pro-inflammatory environment of the aging brain is primarily due to the formation of senile plaques in the form of deposits of amyloid β, and neurofibrillary tangles, which consist of intracellular aggregates of aberrantly phosphorylated tau protein (Jeroen, et al., 2011). Senile plaques are linked to an inflammatory response through an increase in activated complement proteins, cytokines, as well as activated microglia and astrocytes (Akiyama et al., 2000). The stability of the tau protein has critical implications with regard to microtubule dynamics. Tauopathies have been associated with Alzheimer’s disease and frontotemporal dementia, two disease states that have intimately been tied to the aging process (Baird and Bennett, 2013). Tau degradation is specifically known to cause microtubule defects resulting in failure of cellular machinery responsible for maintaining normal neuronal transport (Tang-Schomer et al., 2012). Site-specific microtubule dysfunction results in focal buildup of proteins, including APP, causing axonal inflammation due to transport interruption, causes recruitment of activated microglia to the site, which can result in detrimental effects such as the release of pro-inflammatory cytokines, furthering the detrimental process of neuroinflammation (Tang-Schomer et al., 2012).

Neuronal atrophy is believed to be a normal part of the aging process through the loss of cytoplasmic proteins causing the overall cell size to decrease (Draganski et al., 2013). In the brain, such atrophy can result in neuronal loss and cortical network dysfunction through loss of synapses. Neuronal loss and network dysfunction have
shown a close association with dementia, a disease state that primarily manifests as a consequence of age, in the form of progressive impairment of memory and intellect, alterations to social behavior, visual-spatial perception and aphasia (Ford, 2014). Given the compromised state of the cell due to age alone, TBI could profoundly exacerbate such existing pathologies, resulting in a much poorer prognosis for the elderly post-injury.

Classifying TBI

Though the term TBI encompasses a complex and multifactorial disease, at the most general level, components can be broken down into either primary or secondary injury depending on when the injury occurs (Adams, 1992; Osborn et al., 2009). Primary injury refers to the initial damaging insult, which directly affects brain tissue at the time of injury. Secondary injury is due to the initiation and evolution of cellular and molecular cascades, which become activated due to the primary injury (Adams, 1992; Weber, 2012). Both primary and secondary processes are known to occur following either of the major brain injury classifications, focal or diffuse TBI, though the extent of involvement and specific roles of these processes in either classification may differ significantly. It is also worth noting that focal or diffuse injuries are not necessarily autonomous forms of injury, but may occur concomitantly depending on the type of trauma, particularly at more severe levels (Lovell and Franzen, 1994; Smucker et al., 2009). The combinatorial effect of both focal and diffuse injuries has been recognized as a major determinant in patient outcome (Povlishock and Katz, 2005; Osborn et al., 2009). Additionally, recent work has shown a depletion of transverse bands as a normal consequence of age (Shepherd et al., 2012). The loss of transverse bands has shown direct involvement with
the AIS, as the para-AIS, the first myelinated region of the axon, becomes structurally compromised resulting in structural alterations to the AIS (Shepherd et al., 2012). The loss of transverse bands at the para-AIS formed the rationale behind choosing the AIS as a cellular domain to which the current study is focused on.

**Focal TBI**

Focal TBI is commonly associated with an injury in which the head strikes or is struck by an object, commonly seen in the form of a penetrating head injury (Gennarelli and Graham, 2005). This form of trauma derives from the brain coming in direct contact with a foreign object or the inner surface of the cranial vault, causing immediate damage to brain parenchyma and vasculature at the site of injury (Lighthall, 1988; Saatman and Duhaime, 2008). Due to the nature of the mechanism for injury, focal TBI often is associated with contusions and hemorrhage/hematoma formation within the brain (Granacher, 2007).

In a clinical setting, contusions are often presented as superficial cortical bruising with more severe injuries spreading into subcortical white matter (SCWM) (Graham, 1996; Hayden et al., 2007). Though the precise mechanisms behind contusional pathogenesis are still largely unclear, it is believed that local contact phenomena and/or shearing forces initiate petechial bleeding within neocortical grey matter or at grey/white interfaces, helping to facilitate neuronal loss and ischemic consequences (Povlishock and Katz, 2005; Rehman et al., 2008). Contusional changes have been associated with cellular membrane degradation (Scalea, 2005) pathologic ionic influx into the cell (Weber, 2012), and the exhaustion of local cellular energy deposits, ultimately leading to
programmed cell death and the formation of a necrotic cavity at the site of the lesion (Graham, 1996; Scalea, 2005; Luo et al., 2011).

In addition to contusion, hemorrhages and hematoma formation induced by TBI can appear in a variety of forms (Granacher, 2007). Clinical presentation can often be in the form of subdural hematomas, commonly caused by tearing of subdural bridging veins connecting the dura mater and arachnoid mater. Epidural hematomas are typical of skull fractures, which result from a rupturing of meningeal arteries causing bleeding between the dura mater and the inner cranial vault. Additionally, subarachnoid hemorrhage and/or intracerebral hemorrhages can also be present following focal TBI (Granacher, 2007).

Within the context of aging, focal TBI-related bleeding could have critical implications as a high percentage of older people use medications (e.g. anticoagulants and/or anti-platelets), which may worsen cerebral damage after trauma (Stocchetti et al., 2012).

**Diffuse TBI**

Distinct from focal injuries, diffuse TBI occurs over a widespread area and is not limited to a specific injury site caused by direct impact. Because of the widespread nature, neurologic deficits, including loss of consciousness (LOC), cognitive impairments, and neuropsychiatric issues, can be less specific than symptoms typically seen in focal TBI (McAllister, 1992; Stocchetti et al., 2012). Diffuse TBI results in scattered damage at multiple foci throughout the brain and is most commonly associated with accelerating/decelerating injuries (Gennarelli and Graham, 2005). During these injuries, various tissue types with differing densities accelerate at different rates, which can result in rotational and translational shear and tensile forces being transmitted
through the brain (Graham, 1996; Shepherd, 2004; Osborn et al., 2009). The transmission of these forces cause cells and their processes as well as microvasculature to be subject to different levels of strain, resulting in different levels of injury (Smith et al., 2003). Due to the varying degrees of injury, a wide range of cellular responses has been known to occur (Singleton et al. 2002, Baalman et al., 2013, Greer et al. 2013). Additionally, underlying the diffuse nature of the injury, uninjured neurons can be found directly adjacent to injured neurons throughout the brain parenchyma (Povlishock et al., 1983; Adams et al., 1989; McGinn et al., 2009; Greer et al., 2012; Greer et al., 2013). Further, as distinct regions of the brain show a wide range of compositional diversity, various areas may react differently to straining or shearing forces. Certain areas can be affected preferentially depending on the type of tissue and its tolerance to withstand these forces (Singleton and Povlishock, 2004; Greer et al., 2012; Greer et al., 2013). It has been shown that even within these specified regions, individual cells can be distressed differentially (Singleton et al., 2002; Greer et al., 2013).

The term diffuse brain injury is broad and covers a spectrum of injuries including meningitis, hypoxia-ischemic injury, diffuse swelling or edema, diffuse vascular injury, diffuse neuronal somatic injury, and diffuse axonal injury (Gennarelli and Graham, 2005). The different forms of diffuse TBI often occur in tandem with focal TBI and have been shown in a variety of experimental models, which is beyond the scope of the current communication. For the purposes of this study, we will limit our focus to one form of diffuse TBI, specifically diffuse axonal injury.
**Diffuse Axonal Injury**

*Epidemiology and Background*

Diffuse axonal injury (DAI) is believed to be the most prevalent and significant pathological component in mild, moderate, and severe traumatic brain injury (Povlishock, 1992; Maxwell and Graham, 1997; Smith and Meaney, 2000; Iwata et al., 2004). The overall extent of axonal damage is known to be a major contributing factor to posttraumatic morbidity and mortality with a strong correlation to the degree of functional deficits (Adams et al., 1982; Povlishock, 1992; Iwata et al., 2004). Each year, an estimated 26,000 deaths occur due to DAI (Meythaler et al., 2001). DAI is one of the primary factors underlying unconsciousness and persistent vegetative state following TBI and is believed to be present in roughly half of all severe head injuries (Wasserman and Koenigsberg, 2007). Based on neuroimaging research, the duration of coma or LOC correlates to the severity of DAI (Takaoka et al., 2002).

*Detection and Clinical Diagnosis*

Historically, much of the early work detailing DAI was confined within the context of severe TBI; however, as our understanding of the injury has progressed, the nomenclature has now been broadened to include mild to moderate TBI as well (Adams et al., 1989). Even today, clinical diagnosis of DAI is not without its challenges. Despite the use of modern imaging modalities such as computerized tomography and magnetic resonance imaging (MRI), DAI diagnosis can be difficult due to the microscopic nature of the injury. Clinicians often look for diagnostic clues in the form of punctate micro-
hemorrhagic lesions that accompany DAI to make an inferential diagnosis (Osborn et al., 2009). Advances in MRI technology, however, have the potential to aid in the detecting and diagnosing of DAI. Specialized sequences such as diffusion tensor imaging (DTI) have enabled the use of fractional anisotropy maps to document the integrity and direction of white matter tracts (Inglese et al., 2005) and DTI “tractograms” have allowed for the delineation of white matter tract disruption patterns (Osborn et al., 2009). Recently, high-resolution DTI has been able to provide unique insight into changes in cortical microstructure and was instrumental in the Human Connectome Project for mapping human brain connections and the vulnerability (Sotiropoulos, et al., 2013). Another specialized MRI sequence is susceptibility-weighted imaging, which has been able to confirm discrete DAI foci following even mild TBI (Osborn et al., 2009; Yuh et al., 2012). For micro-hemorrhagic lesions, susceptibility-weighted imaging is recommended as the preferred MRI sequence (Osborn et al., 2009).

**Experimental Modeling of DAI**

Despite advances in modern medicine and biomedical research, there is no “cure” for DAI and no specific treatment beyond the standard protocol for care following any type of head injury (Osborn et al., 2009). Due to its pervasive nature, contribution to subsequent morbidities, and lack of real treatment, DAI has long been the focus of experimental models aimed at uncovering potential therapeutic strategies (Sharp et al., 2014). In early stages, DAI models utilized low order primates to undergo induced traumatic coma by rapid multi-directional acceleration without impact in several directions. The comatose period, level of neurologic impairment, and location and
amount of DAI were all measured (Gennarelli et al., 1982). Gennarelli and colleagues found that using duration of coma and quality of outcome as markers for severity of injury, DAI was directly proportional to the degree of injury, suggesting the importance of DAI to morbidities associated with TBI. In 1976, mechanical brain injury was produced in cats using a fluid percussion model to induce elastic deformation and produce DAI (Sullivan et al., 1976). The novel fluid percussion model proved advantageous over previous models with regard to the ease in which a transient pulse could be measured and controlled thereby producing a consistent injury as well as permitting the study of more subtle changes following mechanical brain injury without intraparenchymal hemorrhage or subarachnoid hemorrhage (Sullivan et al., 1976).

In light of these advantages, fluid percussion injury was later used to demonstrate DAI occurrence post-injury within efferent fibers of the brainstem using the same feline model (Povlishock et al., 1983). By using peroxidase-laden gels to label axons, Povlishock and colleagues (1983) demonstrated that DAI could produce eventual secondary axotomy. Importantly, prior to axonal transection, intra-axonal swellings were observed within continuous axons due to the accretion of horseradish peroxidase (HRP). HRP swellings continued to grow, eventually becoming associated with accumulations of other cellular entities such as mitochondria and neurofilaments. It was found that these swellings eventually resulted in the axonal disconnection. Intra-axonal swelling eventually leading to disconnection proved to be a landmark discovery in DAI, as it was the first study to suggest molecular cascades were responsible for transection, thus introducing the new concept of progressive secondary axotomy (Povlishock et al., 1983).
Many different models have been utilized for DAI studies ranging from axonal transection, crush, and stretching to mechanical TBI (Barron, 1983; Gennarelli et al., 1992; Iwata et al., 2004). Though valuable information can certainly be gleaned from such work, total understanding of the mechanisms involved with DAI are complicated due to the fact that physical transection does not replicate the progressive axonal failure described in DAI (Povlishock, 1992). Moreover, many of the current mechanical models used in TBI frequently cause disruptive lesions involving contusion or hemorrhage that significantly complicate the evaluation of generalized neuronal alteration and/or circuit disruption in response to DAI (Povlishock and Katz, 2005). Given that mild DAI occurs within a relatively intact CNS microenvironment uncomplicated by hemorrhaging/contusion, inflammatory responses, or substantial reactive astrocytic gliosis (Singleton et al., 2002; Kelley et al., 2006; Greer et al., 2011), central fluid percussion injury (cFPI) was chosen as the ideal model to facilitate the current investigation.

Pathobiogenesis Following DAI

Based on the work of Povlishock and colleagues, the clear association between progressive secondary axotomy and DAI in mild to moderate TBI has been established (Povlishock, et al. 1983). As investigations have continued to shed light on these secondary processes behind DAI, new information suggests that certain mechanisms may be site-specific and differ based on location of injury within the neuron. Further, certain loci within the neuron may be more vulnerable to injury than others (Greer et al., 2013). As novel findings continue to surface, certain features may play an increased or
diminished role in the future and entirely new mechanisms may be discovered; however, as of now, the following are the major characteristics of pathobiogenesis associated with DAI.

**Axolemmal Mechanoporation and Disruption of Ionic Homeostasis**

As previously discussed, shearing forces in DAI do not typically cause the primary disconnection of axons and this phenomenon is generally observed only in severe cases (Povlishock et al. 1993; Osborn et al., 2009). The majority of trauma-induced axonal pathology is a secondary, progressive process that involves a series of deleterious molecular cascades (Singleton et al., 2002; Iwata et al., 2004; Greet et al., 2013). It is generally accepted that a primary element of DAI pathobiogenesis is the immediate increase in intra-axonal calcium (Ca$^{2+}$) levels at the site of injury causing a disruption in ionic homeostasis (Fineman et al., 1993; Maxwell et al., 1995; Iwata et al., 2004; Weber et al., 2012). Though consensus exists with regard to pathologic increases in intra-axonal Ca$^{2+}$, the source from which the Ca$^{2+}$ came is still a topic of ongoing discussion. It is postulated that the primary source is extracellular, with rapid, mechanical stretching of axons inducing extracellular Ca$^{2+}$ influx through the axolemma (Smith et al., 1999; Weber et al., 2012). Other potential sources are intracellular and include the release of Ca$^{2+}$ stores from damaged mitochondria (Nicholls, 2009) and/or the smooth endoplasmic reticulum (SER) (Ouardouz et al., 2003; Weber et al., 2012).

Extracellular Ca$^{2+}$ is believed to rush into the axon after injury due to immediate and ephemeral axolemmal mechanoporation. This process occurs via the formation of pores within the axolemma due to deformations in the tissue caused by the mechanical
strain of the injury (Gennarelli, 1993; Iwata et al., 2004; Greer et al., 2011). Evidence supporting this theory has been shown in vitro in biaxial dynamic stretch injury models, where small molecular weight tracer (~400 Da) uptake was observed following induced mechanoporation with coinciding Ca\(^{2+}\) influx that was not abolished following ion channel blockage (Geddes-Klein et al., 2006; Kilinc et al., 2009). Further evidence for axolemmal permeability has come from in vivo research where uptake of the larger molecular weight species were observed at damaged focal regions along the axon (Pettus and Povlishock, 1996; Singleton and Povlishock 2004; Farkas and Polvishock, 2006; Kelley et al., 2006).

To emphasize the complexity of the injury, mechanoporation does not always appear to be the primary mechanism. Following mild TBI, DAI has been shown to occur without the presence of axolemmal disruption through tracer uptake (Pettus et al., 1994). Axons demonstrating impaired axonal transport, progressive swelling, and cytoskeletal alterations in the form of neurofilament misalignment ultimately became disconnected, with no observed evidence of tracer uptake. It is important to note, however, the tracers used were much larger (3-4 kDa) than the ions (20-40 Da) believed to be responsible for the initiation of molecular cascades in DAI.

Channelopathy

Because voltage gated sodium channels (Na\(_V\)) have been shown to be vulnerable to mechanical stress (Tabarean et al., 1999) it was originally proposed that injury induced alterations to ion channels could be the initiating factor behind Ca\(^{2+}\) influx (Wolf et al., 2001). Following in vitro dynamic mechanical stretch injury in axons, Wolf and
colleagues showed that Ca\textsuperscript{2+} influx occurred in two phases: first a large immediate transient increase followed by minor incremental elevations thereafter. Following pretreatment with tetrodotoxin, a neurotoxin known to cause Na\textsubscript{V} blockage, elevations of intra-axonal Ca\textsuperscript{2+} were halted. Further, intra-axonal Ca\textsuperscript{2+} levels were then diminished following blockage of Na\textsubscript{V} and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers, lending evidence to support the theory that alterations to Na\textsubscript{V} channels may be the initial source of elevated intracellular calcium (Wolf et al., 2001; Staal et al., 2010). Additional stretch injury models have also shown that calpain-dependent proteolytic cleavage of the \(\alpha\)-subunit of Na\textsubscript{V} channels occurs following injury resulting in persistent Na\textsubscript{V} permeability causing a feed-forward pathway of sustained intra-axonal Ca\textsuperscript{2+} elevation (Iwata et al., 2004; von Reyn et al., 2009).

Despite this evidence, the precise role of Na\textsubscript{V} pathology following DAI is still unclear. Though calpain inhibition has been shown to attenuate Ca\textsuperscript{2+}, it does nothing to prevent the initial spike that is observed following injury. In light of this, it is now believed Na\textsubscript{V} pathologies, in the form of either injury-induced mechanical alteration or proteolytic cleavage, are a secondary process that continue to exacerbate ionic disruption, resulting in persistent elevations to intra-axonal Ca\textsuperscript{2+} levels (Iwata et al., 2004, von Reyn et al., 2009).

**Mitochondrial Damage and Dysfunction**

As mitochondria perform many key functions such as cellular energy production, Ca\textsuperscript{2+} buffering/removal from the axoplasm, and apoptosis initiation (Krieger and Duchen, 2002), their role within the context of DAI pathobiogenesis is central (Büki et al., 2000;
Starkov 2010; Weber, 2012). DAI studies using various animal models have observed morphological changes to mitochondria including mitochondrial swelling, membrane blebbing, and ruptured cristae (Pettus and Povlishock, 1996; Büki et al., 2000; Smith and Meaney, 2000; Weber, 2012). These morphologic changes to mitochondrial structure are consistent with pathological progression of local Ca^{2+} overloading that results in the release of cytochrome-c, with the activation of caspase, which has been shown to participate in axonal disconnection (Büki and Povlishock 2006; Starkov et al., 2010). Ca^{2+} overloading and mitochondrial perturbation has also been closely tied to the opening of the mitochondrial permeability transition pore (mPTP). The opening of the mPTP permealizes the mitochondrial membrane to larger molecules, leading to the uptake of water, causing mitochondrial swelling and eventual rupture to occur (Hirsch et al., 1998; Forte and Bernardi, 2005). Significant mitochondrial damage caused by DAI can lead to bioenergetic failure, which can continue to further Ca^{2+} influx due to ATP-dependent Ca^{2+}-ATPase and Na^{+}-ATPase pump failure furthering the process of pathobiogenesis (Forte and Bernardi, 2005; Starkov et al., 2010).

SER Dysfunction and Excitotoxicity

Similar to mitochondria, one of the functional roles of the SER is to buffer intra-axonal Ca^{2+}, which is accomplished through a number of ion pumps located on the SER membrane. As a buffering source, the SER contains intracellular Ca^{2+} stores, and is believed to play a potential role in elevated Ca^{2+} concentrations following DAI (Weber, 2012). Under physiologic conditions, the SER removes excess Ca^{2+} from the axoplasm
via ATP-dependent SER Ca\textsuperscript{2+}-ATPases (SERCA), pumping ions into SER for storage. Following a decrease in normal intra-axonal Ca\textsuperscript{2+} levels, the SER can release Ca\textsuperscript{2+} from its stores through the use of various ion pumps, including inositol triphosphate receptors (InsP3R) (Stirling and Stys, 2010). Both SERCA and InsP3R pumps are known targets for proteolytic cleavage by Ca\textsuperscript{2+}-dependent cysteine proteases, rendering them nonfunctional and potentially causing further elevation of intra-axonal Ca\textsuperscript{2+} levels (Salamino et al., 1994; Assefa et al., 2004; Weber, 2012).

Following DAI, excitotoxicity is also believed to play a contributory role in pathologic elevation of intra-axonal Ca\textsuperscript{2+} levels through a process that is closely tied to the SER. Extracellular glutamate and other excitatory amino acids have been shown to increase following TBI (Hinzman et al., 2010). A pathologic post-injury increase in these signaling molecules is believed to continually activate cell surface receptors. Excessive activation of these receptors has been shown to promote secondary injury cascades that mediate SER Ca\textsuperscript{2+} release into the axoplasm, contributing to further ionic instability (Stirling and Stys, 2010).

Caspase-3-Mediated Proteolysis

Following DAI and subsequent Ca\textsuperscript{2+} dysregulation, activation of Ca\textsuperscript{2+}-dependent cysteine proteases has been observed. Caspase-3, while traditionally associated with programmed cell death, has been observed following DAI independent of apoptosis (Stone et al., 2002; Saatman et al., 2010). Mitochondrial damage associated with Ca\textsuperscript{2+} induced mPTP formation has been shown to cause the release of numerous proteins from
the intermembrane spaces, in particular cytochrome-c (Büki et al., 2000; Galluzzi et al., 2009). Once released into the axoplasm, cytochrome-c accumulates and eventually activates caspase-3. Once activated, caspase-3 activity has been correlated to continual degradation of the intra-axonal cytoskeleton through the cleavage of spectrin molecules and organelle disruption (Wang et al., 1998; Büki and Povlishock, 2006; Galluzzi et al., 2009). Both of these deleterious events are known to be participatory processes leading to eventual axonal disconnection (Büki et al., 2000; Büki and Povlishock, 2006). Moreover, evidence suggests that caspase-3 is also responsible for the cleavage of calpastatin, a calpain protease inhibitor, which could lead to the enhancement of damaging calpain-mediated proteolysis (Wood and Newcomb, 1999; Huang et al., 2014).

**Calpain-Mediated Proteolysis**

Mitochondrial damage and mPTP formation have also been linked to the activation of a second Ca\(^{2+}\)-dependent cysteine protease, calpain (Saatman, 2010). Two predominant isoforms of calpain are known to activate depending on Ca\(^{2+}\) concentration: µ-calpains and m-calpains. Both isotypes play important roles in normal physiologic conditions, including synaptic plasticity, protein breakdown, and cell signaling (Wu and Lynch, 2006). Under pathologic conditions, calpain has been demonstrated to play a role in the degradation of numerous axonal components, including cytoskeletal and scaffolding proteins, such as alpha-II-spectrin, Ankyrin G (ankG), and microtubule associated protein tau (Büki et al., 1999; Reeves et al., 2010; Saatman, 2010). Following injury, calpain has also been shown to activate caspase-3 (Nakagawa and Yuan, 2000; Huang et al., 2014). Given that caspase-3 is responsible for the cleavage of calpastatin,
as previously discussed, there appears to be cross-talk between the two cysteine proteases, which could intensify proteolysis and axonal damage (Wang et al., 1998; Blomgren et al., 2001; Greer et al., 2011).

_Cytoskeletal Proteolytic Degredation_

As mentioned before, calpain and caspase-3 have a wide range of target proteins. Some proteins, such as alpha-II-spectrin, can be the target of either, which has been shown experimentally with evidence of distinct spectrin breakdown products depending on the specific protease responsible for cleavage (Wang, 2000). Acting together, the two cysteine proteases can result in microtubule loss, direct actin degradation, pathologic alterations to neurofilaments, and damage to scaffolding proteins, such as ankyrinG (ankG) (Maxwell and Graham, 1997; Yoshida et al., 1984; Schafer et al., 2009). AnkG is of chief concern in this current communication due to its unique localization within the nodes of Ranvier and the axon initial segment (AIS) and the importance of its maintenance therein.

_The Axon Initial Segment_

The axon itself can be delineated into specific, functionally distinct domains. These different domains are the AIS, the internode, the juxtaparanode, the paranode, and the node of Ranvier (Figure 1.1) (Salazar, 1997). Though numerous studies have shown involvement within the nodal, paranodal, juxtaparanodal, and internodal regions following DAI (Erb and Povlishock, 1988; Pettus and Povlishock, 1996; Maxwell and Graham, 1997), more recent work by Greer and colleagues has demonstrated that the AIS
specifically shows a predilection for traumatically induced axonal damage, with an
overwhelming majority of swellings occurring in direct association with the AIS
compared to other axonal regions (Greer et al., 2013). In light of these findings, the AIS
domain was chosen as the specific focus of the current investigation.
Figure 1.1 Axonal domains. The AIS is directly adjacent to neuronal soma (red). The paranode (orange arrow) is the region directly adjacent to the Node of Ranvier. The juxtaparanode (green arrow) is directly adjacent to the paranode, and the internode (blue arrow) is directly adjacent to the juxtaparanode. Adapted from OpenStax, 2012.
AIS Structure and Composition

The AIS is the most proximal unmyelinated region of the axon, adjacent to the neuronal somata and is responsible for the initiation of action potentials (AP) and maintaining cell polarity (Figure 1.2) (Buffington and Rasband, 2011). The specialized electrical properties of the AIS are primarily due to the clustering of NaV at the proximal and distal ends of the domain (Caldwell et al., 2000; Colbert and Johnston, 2006). AP initiation is primarily associated with NaV1.6 at the distal end of the AIS (Buffington and Rasband, 2011). The other predominate isoforms, NaV1.2/1.1, are generally found clustered in the proximal region and mainly contributes to somatodendritic AP backpropogation (Hu et al., 2009). AP initiation is accomplished through depolarization, a process by which ions flow through voltage gated channels, resulting in a change in membrane potential. Once sufficient depolarization has occurred to reach the intrinsic threshold of the cell, an all-or-nothing electrical spike occurs resulting in the initiation of an AP (Peles and Salazar, 2000). For this process to occur a high Na⁺ current and low AP threshold is necessary, which is achieved by precise clustering and alignment of NaV (Buffington and Rasband, 2011). The process of precise clustering and alignment is feasible only through NaV anchoring to the highly stable, cytoskeletal scaffolding protein, ankG (Rasband, 2008).
**Figure 1.2 The axon initial segment.** Clustering of Nav 1.6 primarily occurs at the distal end and is responsible for the initiation of the AP. AnkG is a cytoskeletal scaffolding protein responsible for anchoring numerous membrane bound proteins. AnkG is tethered directly to βIV spectrin which is connected to the actin microfilament cytoskeleton.
AnkG has been referred to as the “master organizer” of the AIS (Rasband, 2010). Experiments have shown through genetic ablation that ankg is required for proper clustering and placement of numerous AIS membrane proteins (Zhou et al., 1998). In addition to Na\textsubscript{v} ankG is responsible for anchoring voltage gated potassium channels (K\textsubscript{v}), neurofascin-186 (NF-186), neuronal cell adhesion molecule (NrCAM) (Rasband, 2010). AnkG is connected to the actin-based cytoskeleton through βIV-spectrin tethering (Buffington and Rasband, 2011). Due to its extensive anchoring motif, ankG plays an important role in AIS stability and is required for formation and maintenance of normal neuronal polarity, which has been shown to be a functional prerequisite (Rasband, 2010).

**AIS responses following injury**

Little is known about the exact injury-induced mechanisms that follow DAI in the AIS, particularly with regard to age. Following mild cFPI, minor cytoskeletal disruptions in the form of AIS shortening evidenced by ankG immunolabeling were observed (Greer et al., 2013). The consistent shortening was linked to a delayed restoration of neuronal excitability. Despite the fact that 60% of all swellings were directly linked to the AIS, which was presumed to be more biomechanically vulnerable, very little pathobiogenesis was observed, at least in the conventional DAI mechanisms discussed above. Greer and colleagues postulate that the mechanisms long associated with DAI may play a diminished role in the AIS and pathogenesis may be occurring via alternate mechanisms (Greer et al., 2013).

Though few studies have been done to characterize AIS responses following DAI, other experimental models may prove useful in better understanding AIS.
pathobiogenesis. Similar to DAI, the pathophysiology of ischemic injury is due, in part, to a large Ca\textsuperscript{2+} influx, which subsequently activates proteolytic calpains (Simon et al., 1984; Shafer et al., 2009). In one stroke model, rapid calpain-mediated proteolysis of the ankG and spectrin AIS cytoskeleton occurred following occlusion of the middle cerebral artery (Shafer et al., 2009). The breakdown of ankG caused Na\textsubscript{V} clusters to deteriorate and subsequent loss of neuronal polarity, ultimately leading to loss of function within the neuron. Importantly, apoptosis was not observed, which is consistent with DAI observations (Shafer et al., 2009). As increased Ca\textsuperscript{2+} has been reported to be a prevalent event in TBI, a similar mechanism could be at play. It is speculated that disruptions to AIS cytoskeletal scaffolding proteins occurs with age (Buffington and Rasband, 2009). It could be within the realm of possibility that these disruptions could weaken the structural integrity of ankG, making the AIS more vulnerable to injury with age.

Only recently it has been discovered that the AIS might play a primary role in the pathobiogenic process and eventual axonal disconnection following DAI (Greer et al., 2013). Though this process has been qualitatively characterized in young animals, the actual mechanisms are still being determined. Further, as an epidemiological shift occurs toward the elderly suffering from TBI (Stocchetti et al., 2012), the need to further examine these processes, especially within the context of an aging brain, is becoming more evident. With this goal in mind, we set out to better characterize the post-injury structural alterations to the AIS as a consequence of age. Our hypothesis was that the biomechanical vulnerability of the AIS could be a major underlying factor that could help explain why such a strong correlation between age and poor prognostic outcome following DAI existed.
MATERIALS AND METHODS

Animals

Eight twenty-two month old c57/black6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in the Virginia Commonwealth University Division of Animal Resources vivarium. Nine three-month old mice were obtained from a colony of c57/black6 mice maintained in the Virginia Commonwealth University Division of Animal Resources vivarium. Food and water for the mice were provided *ad libitum*. All housed mice were kept on a 12 hour light/12 hour dark cycle. All mice were appropriately housed in an AAALAC certified facility, utilizing protocols in accordance with the standards set by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All procedures were conducted in accordance to the methods outlined and approved by the VCU Animal Care and Use Committee.

Surgical Preparation and Injury Procedure

All surgical procedures and fluid percussion injuries were conducted by Dr. Anders Hänell in the laboratory of Dr. John Povlishock. Briefly, seventeen c57/black6 mice underwent surgical preparation for the central fluid percussion injury (cFPI) procedure. Each mouse was placed in an anesthesia chamber and was anesthetized using 4% isoflurane in 100% O₂. Once anesthetized, the thigh of each mouse was shaved for
intra-operative physiological monitoring and each animal was placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) with anesthesia maintained using a fitted nose cone with 1-2% isoflurane in 100% O₂. 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 housed below the animal and configured to monitor rectal temperature. Normal physiologic homeostasis (pulse rate, respiratory rate, and blood oxygenation) was monitored intra-operatively using pulse oximetry via a thigh sensor (STARR Life Sciences Corp.; Oakmont, PA) excluding any mice displaying a physiological anomaly. To expose the skull, a midline sagittal incision was made from bregma to lambda. Once the exposed skull was cleaned and dried, a 3.0 mm circular craniotomy was performed along the sagittal suture at the midpoint between bregma and lambda, while preserving the underlying intact dura. The surgical hub was then attached to the craniotomy site using a sterile Leur-Loc syringe hub that was cut away from a 20-gauge needle using a cyanoacrylate adhesive. Once a complete seal between the hub and skull was visually confirmed, a layer of dental acrylic was coated around the base of the hub to reinforce correct positioning of the hub, helping to ensure the stability of the hub during injury. After the dental acrylic had cured, the scalp surrounding the hub was sutured and topical bacitracin and lidocaine ointment were applied to the site to prevent bacterial infection. The mouse was then removed from anesthesia, placed in a warm cage, and monitored until fully ambulatory (approximately 60-90 minutes). For cFPI, each mouse was re-anesthetized using 4% isoflurane in 100% O₂. The male end of a spacing tube was then fixed to the hub with the saline filled female end of the hub-spacer assembly 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 +/- 0.04 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. The peak pressure of each pressure pulse was measured using a transducer and displayed on a storage oscilloscope (Tektronix 5111, Beaverton, OR). Post-injury, the mice were observed until spontaneous respiration resumed. Both hub and dental acrylic were then detached together and the incision was immediately sutured prior to the animal emerging from anesthesia/unconsciousness. Once sutured, topical bacitracin and lidocaine ointment were then administered to the closed scalp incision site. To test the duration of transient unconsciousness, response time to the following reflexes were tested: toe pinch, tail pinch, corneal blink, pinnal, and righting. Following response to the righting reflex, animals were then moved to a warmed cage to assure maintenance of normothermia and monitored until recovery was confirmed before being moved back to the vivarium. All of the above procedures were performed for sham injured animals, except the release of the pendulum on the fluid percussion apparatus. Severity of the fluid percussion injury was verified by analyzing righting reflex recovery times with a two-way ANOVA with a Turkey’s HSD post hoc analysis.

**Animals Utilized for Study**

C57/black6 male mice were used for both aged and young groups. Aged mice were 22 months old and young mice were 3 months old. Both groups were allowed to recover for predetermined times of 3 or 24 hours following injury (sham aged, n=2; 3hr
aged, n=3; 24hr aged, n=3; sham young, n=3; 3hr young, n=3; 24 hr young, n=3). Sham mice were allowed to recover for 24 hours (Table I).
<table>
<thead>
<tr>
<th>Group</th>
<th>Young Sham</th>
<th>Young 3-hour</th>
<th>Young 24-hour</th>
<th>Aged Sham</th>
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<td>$n =$</td>
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Table I. $n$ numbers for age/injury groups used in study
Tissue Preparation for Double Label Immunofluorescence

Mice were allowed to recover for specific predetermined times post-injury (see Animals Utilized for Study). At the two fixed survival times, animals were intraperitoneally injected with an overdose of 2’2’2’ tribromoethanol (avertin). Initially, a primary incision was made from abdomen to sternum with two subsequent incisions made from sternum to each axilla. The diaphragm was then bisected and the rib cage was excised, exposing the heart. An incision in the right atrium allowed for an exit point for the blood and perfusate. A 22 gauge, 1 inch needle was then carefully inserted into the left ventricle, making sure the interventricular septum and aorta were not punctured. Mice were then flushed with 0.9% NaCl for 5-7 minutes or until the perfusate appeared clear. After the perfusate flowed clear, the mouse was transcardially perfused with 0.1 M 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.

The entire 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. After 48 hours of incubation, brains were removed from PBS-containing sucrose solution, the olfactory bulbs were then removed and the remaining brain was frozen in Optimal Cutting Temperature (OCT) compound and sectioned at 40 µm using a Leica CM 1850 cryostat. 15 sets of six 40 µm sections were collected from cortical regions. Sections were collected from -2.5 mm posterior to bregma to 1.1 mm anterior to bregma (Paxinos and Franklin, 2003).
Double Label Immunohistochemistry Protocol

Primary antibodies were polyclonal rabbit anti-amyloid precursor protein (APP) (C-Terminus) (1:500; Invitrogen) and AnkyrinG (AnkG) (1:200; NeuroMab Facilities). Routine immunostaining protocol is as follows: slides were taken from -80°C to and allowed to dry at room temperature before removing any excess OCT compound from the perimeter of the slide. A hydrophobic barrier was then drawn around the tissue using a PAP Pen (Super PAP Pen, Electron Microscopy Sciences) to ensure solutions would remain in contact with the tissue sections. Slides were then placed in three 5 minute rinses in PBS, followed by a 2 hour incubation with a blocking solution. The blocking solution contained ~5% cold water fish gelatin, 0.5% Triton X-100, and 1.25 mL of Mouse on Mouse Kit, MOM™ (Vector Laboratories; Burlingame, CA). The MOM kit was used to block endogenous IgG, as the AnkG primary antibody was derived from a mouse host. Slides then underwent three 10 minute washes in ~5% cold water fish gelatin and 0.5% Triton X-100 in PBS. The tissue was then incubated overnight at 4°C in a humidified chamber with the primary antibodies in ~5% cold water fish gelatin and 0.5% Triton X-100 in PBS. On the following day, the tissue underwent three additional washes for 5 minutes each containing ~5% cold water fish gelatin and 0.3% Triton X-100 in PBS. Tissue sections were then incubated in the appropriate Alexia fluor secondary antibody (Alexa 488 and Alexa 594) (Invitrogen; Carlsbad, CA) diluted 1:500 in a solution of ~5% cold water fish gelatin and 0.3% Triton X-100 in PBS. Each slide was then given three rinses in PBS, mounted with Vectashield™ (Vector Laboratories) medium and cover slipped.
Image Collection Using Confocal Microscopy

All images 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. Eighteen fields were collected ninety total sections per mouse. Any mice showing signs of cerebral hemoatoma or subarachnoid hemorrhage as evidenced by blood accumulation were removed from the study. Image collection was restricted to Cortical Lamina V due to the relatively homogenous neuronal population. Cortical Lamina V was established based on prior work using comparable sections stained with BisBenzamide and confirmed using transgenic YFP+ mice. Areas within Cortical Lamina V were then preferentially chosen based on the amount of APP accumulation. To account for auto-fluorescence, spectral profiles for ankG, APP, and lipofuscin were generated and linearly unmixed. A maximum intensity projection was obtained using a 25µm range z-stack optimized for Nyquist sampling at 53 optical planes. A total of 18 images were collected per mouse, with three images taken per section and six sections per slide. The following section contains the settings used for the microscope. All image acquisition and analysis was conducted under the supervision of Dr. Scott Henderson, Director of the VCU Microscopy Facility.
Confocal Microscope Settings

Acquisition Mode:

Objective: EC Plan-Neofluar 40x /1.30 Oil DIC M27  
Scan Mode: Frame  
Frame Size: X = 2076, Y =2076  
Speed: 6  
Averaging: 2  
Mode: Line  
Method: Mean  
Bit Depth: 16 Bit  
Direction: Bidirectional  
Scan Area:  
  Image Size: 212.4 micron x 212.4 micron / pixel  
  size: .10 micron

Channels:

3 Channels total  
Lasers: 488 (3.5%) , 561 (10.8%)  
Pinhole 1.01 Airy Units  
Gain: 619  
Digital Offset: -0.04  
Digital Gain: 1

Z-Stack:

Range: 25.02 microns  
Slices: 53 Optical Slices  
Interval: 0.48  
*Optimal (Nyquist Sampling) for 25 micron sections

Image Analysis

To analyze collected images, AIS Analyzer (Dr. Anders Hänell), Volocity (Perkin-Elmer, Waltham, MA) and ImageJ (NIH) programs were compared to determine which package provided the more efficient and accurate image analysis. After comparison, ImageJ software was used for image analysis. Using ImageJ software, the AIS lengths were measured in pixels and then converted to microns. Rectification of curves was done by manually tracing each AIS using the freehand tracing tool of ImageJ.
Any AIS in contact with an edge was excluded from study. All nodes of Ranvier were also excluded based on a visual analysis of morphology and size. Successful exclusion of nodes was confirmed during analysis as no AIS measurements were less than 5 µm. Though fracturing appeared to be more common in both aged groups, it is not reflected in population profiles in the form of an increase in the percentage of shorter AIS length distributions. If clear orientation was present without substantial separation between fragmented pieces, individual fragments of the same AIS were traced together in a continuous line to yield a single, overall length (Figure 2.1). AIS measurements were collected as actual values (mean +/- SEM) then converted to percentile ranges. Measurements were collected as actual values, segregated into specific length categories and then converted to a percentile range as a distribution of the total AIS population. A 2-way ANOVA test using multiple comparisons was done to measure statistical significance between groups of AIS populations ($p < 0.05$). A 2-tailed t-test was used to compare values for righting reflex times for significance ($p < 0.05$). All statistical analysis was carried out using Prism 6 (GraphPad Software, Inc.).

Additionally, three images per section per 3 hour survived injured mouse were captured using the Zeiss AxioImager1 fluorescence microscope. Image location was determined using AnkG labeling and individual images capturing the red (AnkG) and green (APP) were collected. Using these images the average number of APP accumulations in cortical layer V of young sham, young injured, aged sham and aged injured mice was compared.
Figure 2.1 Protocol used for tracing AIS. Breaks in AIS with clear orientation were connected. Protocol accounted for curvature of AIS.
RESULTS

Pathophysiologic Findings Show Significant Suppression of Righting Reflex Following cFPI in Aged Mice

The overriding hypothesis for this study was that aged mice would exhibit a significantly greater axonal burden following mild traumatic brain injury as compared to young injured mice. The initial comparison between the 2 age groups resulted from the evaluation of the return of their righting reflex following surgery/injury. Ephemeral suppression of the righting reflex in all animals that underwent mild cFPI was 5.8 ± 1.0 min and significantly longer than 2.0 ± 0.3 min for sham-injured animals ($t$ test, $p < 0.05$). Sham animal recovery time was consistent with that of a normal recovery from anesthesia induced by 4 min in 4% isoflurane, denoting that the recovery time was not influenced by the sham-injury. No significant difference was observed in righting reflex between 3-hours and 24-hours survival time groups subjected to cFPI with values of 4.4 ± 1.0 min and 7.4 ± 1.5 min, respectively, suggesting that the severity of the injury was relatively uniform in each group ($t$ test, $p > 0.05$). A significant difference in righting reflex was observed between young, injured animals with a value of 1.4 ± 0.3 min and aged, injured animals with a value of 7.8 ± 0.9 min, indicating aged mice took significantly longer to recover post-injury ($t$ test, $p < 0.0001$) (Figure 3.1). No significant difference was observed between young and aged sham-injury groups with corresponding values of 2.4 ± 0.6 min and 1.7 ± 0.2 min, respectively, indicating that the delayed recovery time observed in aged injured groups was due to cFPI insult and not age alone ($t$ test, $p > 0.05$).
Aged Injured Mice Exhibit Increased Axonal Burden Following cFPI as Compared to Comparably Injured Young Mice

Since the number of APP accumulations in the brain following DAI is a recognized indicator of axonal damage and prognosis (Blumbergs, et al., 1995), the relative prevalence of APP+ swellings was compared between young and aged mice. Since APP swellings undergo dissociation by 24 hours, this analysis was limited to the 3 hour survival group. As shown in Figure 3.2, the frequency of APP+ swellings was elevated in the aged sham mice as compared to the young sham injured animals. Consistent with previous studies, the frequency of APP+ swellings also increased following injury for both young and aged mice; however, the aged mice exhibited a substantial elevation as compared to the comparably injured young animals (Figure 3.3). These findings suggest that axonal burden is increased following injury in the aged brain. Since Greer et al. (2013) previously reported that these swellings have a predilection for the AIS, we confined our subsequent studies to this axonal domain.
Figure 3.1 Number of APP accumulations per field increases with both age and injury.
Figure 3.2 Number of APP accumulations rose with age and injury. Young sham mice (A) had fewer APP accumulations than aged sham mice (B). Young 3-hour injured mice (C) had fewer accumulations than aged 3-hour injured mice (D).
Macroscopic and Microscopic Pathobiogenesis Associated with cFPI-induced Trauma

Immunohistochemical labeled tissue sections from animals subjected to cFPI displayed macroscopic and microscopic features consistent with previous studies of induced mild to moderate cFPI in rat and mouse models (Dixon et al., 1987; Singleton et al., 2002; Greer et al., 2011). No contusion or cavitation was detected in the dorsal neocortex. The brain parenchyma itself showed no indication of overt hemorrhage with only isolated, punctate lesions observed in the corpus callosum. Limited subarachnoid bleeding was observed over the dorsal convexity in proximity to the site of the hub placement; however, hemorrhaging was confined to this focal location with no further involvement of the subarachnoid compartment. At all survival times in both age groups, the ventricular system maintained a physiologically normal appearance with no evidence of pathologic ventricular enlargement indicative of TBI (Greer et al., 2011).

To positively identify injured neurons in Layer V and determine the extent of AIS damage, immunohistochemical analysis of antibodies against APP, a known marker for traumatic axonal injury (TAI) (Stone et al., 2000) and axonal transport pathology (Gentleman et al., 1993) was used for quantitative and qualitative assessment. Both young and aged sham-injury control groups presented with little to no APP accumulations in axons in the neocortex at 24-hour survival time (Figure 3.3), which was consistent with previous reports (Dixon et al., 1987; Greer et al. 2013). Further, no irregularities regarding AIS shape that would indicate TAI had
Figure 3.3 Representative AIS morphology from sham-injured animals. Young mice AIS populations appeared relatively uniform with moderate, gradual curvature (white arrows, A). AIS in aged mice generally appeared thinner caliber and more sinuous, displaying abrupt contortions (white arrows, B). Limited fracturing was noted in both young and aged mice (blue arrows; A, B) Scale 20 µm.
occurred were noted in either group based on ankG immunofluorescence (Figure 3.4 A, B), a known and routinely used marker for establishing the AIS domain (Schafer et al., 2009; Buffington and Rasband, 2011).

Analogous to earlier studies demonstrating mild cFPI in mice, immunofluorescence of APP was detected at each survival time within the various injury groups (Dixon et al., 1987; Greer et al., 2013). APP swellings detected at 3-hour survival times appeared relatively small, ovoid, and generally morphologically uniform in both young and aged groups (Figure 3.5 A). Both young and aged animals at 24-hours survival time showed continual APP accumulation in axons sustaining damage. APP accretions appeared enlarged, elongated, and morphologically heterogeneous, displaying multi-lobulated axonal swellings (Figure 3.5 B). Heterogeneity of APP swellings within the 24-hour group illustrated the highly variable, temporal progression of axonal transport pathology associated with TAI and was consistent with previous observations (Greer et al., 2013). Overall, in Layer V of neocortical gray matter, regions of axons containing APP accumulations were consistently observed in isolated areas of varying population sizes in all injured animals, at both 3-hour and 24-hour survival times, accentuating the spatial and temporal heterogeneity of pathobiogenesis associated with diffuse axon injury (DAI) and consistent with the cFPI model (Greer et al., 2013).
Figure 3.4 Morphological alterations at 3-hours and 24-hours survival time following cFPI. APP swellings at 3-hours survival time in both young and aged groups generally appeared spheroidal (magenta arrow, A) and were associated with AIS shortening (green arrows, A) when compared to AIS populations with no apparent APP association (white arrows, A). 24-hours survival time showed larger, progressive APP swellings that appeared morphologically diverse (magenta arrows, B). APP swellings were associated with shorter AIS (green arrows, B) when compared to AIS with no apparent association in both young and aged mice (white arrows, B). Scale 20 µm.
APP Accumulation in Damaged Axons Following cFPI is Associated with Shortening of the AIS

3-Hours Survival Time in Young and Aged Mice

Though minor morphological heterogeneity was present in APP swellings within 3-hour injury groups of both young and aged mice, quantitative assessment of initial segments in direct association with APP swellings revealed a significant decrease in length (ANOVA, \( p < 0.05 \)) compared to initial segments with no apparent APP association based on ankG immunoreactivity. In young mice, the mean length of the AIS population with direct APP association was 13.8 ± 0.6 µm, with a mean length of 18.8 ± 0.9 µm corresponding to the AIS population with no apparent APP association. Similar changes in AIS length were also observed in the aged group at 3-hours. In aged animals, the mean length of the AIS population with direct APP association was 14.5 ± 0.7 µm, while the AIS population with no apparent APP association had a mean length of 19.0 ± 0.1 µm.

In order to look at more subtle differences in the change in length, the percent of AIS lengths corresponding to a particular range was plotted and separated into five successive categories (<10 µm, 10-15 µm, 15-20 µm, 20-25 µm, >25 µm) as a percentage of the total AIS population. At 3-hours survival time, percentages of AIS with direct APP association were significantly higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and significantly lower in 15-20 µm and 20-25 µm categories (ANOVA, \( p < .05 \)) (Table II; Table III). The overall shift towards AIS shortening was significant
<table>
<thead>
<tr>
<th>AIS Length</th>
<th>% With APP</th>
<th>% Without APP</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10 µm</td>
<td>24 ± 2.7</td>
<td>1 ± 1.0</td>
</tr>
<tr>
<td>10-15 µm</td>
<td>37 ± 5.2</td>
<td>19 ± 5.1</td>
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<td>26 ± 4.9</td>
</tr>
<tr>
<td>&gt; 25 µm</td>
<td>3 ± 1.4</td>
<td>10 ± 3.1</td>
</tr>
</tbody>
</table>

**Table II. AIS lengths for young mice at 3-hours survival time.** Percentages of AIS with direct APP association were significantly higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and significantly lower in 15-20 µm and 20-25 µm categories (ANOVA, \( p < .05 \)). Data are presented as mean ± SEM.
Table III. AIS lengths for aged mice at 3-hours survival time. Percentages of AIS with direct APP association were significantly higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and significantly lower in 15-20 µm and 20-25 µm categories (ANOVA, \( p < .05 \)). Data are presented as mean ± SEM.
in all length categories, with the exception of the >25 µm group, in both young and aged mice. Most noticeable was the significant increase in the <10 µm group (ANOVA, \( p < 0.0001 \)) in both young and aged animals (Figure 3.6 A, C; Figure 3.7).

When comparing sham AIS populations to AIS populations associated with APP at 3-hours, the same significant shift was observed in young animals (Figure 3.4 A; Figure 3.6 A; Figure 3.8 A). Though not significant in any of the five categories based on a criteria of \( p \leq 0.05 \), all groups with AIS less than >25 µm exhibited \( p \) values \( \leq 0.1 \) in aged animals. Moreover, a similar population shift comparable to young animals was also observed in aged mice (Figure 3.4 B; Figure 3.6 C; Figure 3.8 B). The general distribution of the AIS population with no apparent APP association appeared to mirror the population profiles of the respective sham-injured animals in both young and aged animals with no significant difference in any length category (ANOVA, \( p > 0.05 \)), indicating the sub-population’s AIS length was not affected due to injury (Figure 3.4 A, B; Figure 3.6 A, C; Figure 3.9).

24-Hours Survival Time in Young and Aged Mice

Employing the same approach used to quantify AIS length for the 3-hours survival time, quantitative assessment was also performed on 24-hours survival time groups. In the young mouse, the mean length of the AIS population with direct APP association was 16.2 µm, with a mean length of 19.9 µm corresponding to the AIS population with no apparent APP association. Aged mice also showed similar changes at 24-hours, with a mean length of 15.2 ± 0.3 µm in the AIS population with
Figure 3.5 Representative images for 3-hours and 24-hours survival times for young and aged mice. 3-hour survival time in young mice (A). 24-hour survival time in aged mice (B). 3-hour survival time in aged mice (C). 24-hour survival time in aged mice (D). Scale 20 µm.
Figure 3.6 Significant shift towards shortening in populations associated with APP in both young and aged mice at 3-hours survival time. Data are presented as mean ± SEM. (* p < 0.05; ** p < 0.0001)
Figure 3.7 Shift towards shortening in AIS populations associated with APP compared to sham-injury control at 3-hours survival time. Significant shift towards shortening at 3-hours survival time in AIS populations associated with APP in young mice compared to sham (A). Significant shift towards shortening in <10 µm group and similar shift towards shorter AIS with populations associated with APP in aged mice at 3-hours survival time compared to sham (B). Data are presented as mean ± SEM. (* p < 0.05)
Figure 3.8 No significant difference between AIS in sham-injury and non-APP AIS at 3-hours survival time in young and aged mice. Data are presented as mean ± SEM.
direct APP association and a mean length of 18.9 ± 0.4 µm corresponding to the AIS population with no apparent APP association. As was observed at 3-hours survival time, the AIS lengths with direct APP association were significantly shorter (ANOVA, \( p < 0.05 \)) than the AIS population with no apparent APP association in young and aged animals at 24-hours survival time (Figure 3.6 B, D). Population percentages of AIS with direct APP association were higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and lower in 15-20 µm, 20-25 µm, and >25 µm categories (Table IV; Table V). The population profile shift towards shorter AIS length was significant in all groups under >25 µm (ANOVA, \( p < .05 \)) in aged mice (Figure 3.6 D; Figure 3.10 B). The population shift seen in the young mouse was analogous to shift seen in the aged; however, significance could not be tested due to the low \( n \) number for that specific group (Figure 3.6 B; Figure 3.9 A; Table I).

When sham AIS populations were compared to AIS populations associated with APP at 24-hours, a similar shift towards shortening was observed in young and aged animals. AIS with direct APP association were shorter than initial segments observed in sham animals in young and aged mice (Figure 3.4 A, B; Figure 3.6 B, D; Figure 3.11). As seen in the 3-hour survival time, the general distribution of the 24-hour survival time AIS population with no apparent APP association appeared to mirror the population profiles of the respective sham-injured animals in both young and aged animals with no significant difference in any length category (ANOVA, \( p > 0.05 \)), once again indicating the sub-population’s AIS length was not affected due to injury (Figure 3.4 A, B; Figure 3.6 B, D; Figure 3.12).
Table IV: AIS lengths for young mice at 24-hours survival time. Population percentages of AIS with direct APP association were higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and lower in 15-20 µm, 20-25 µm, and >25 µm categories. Data are presented as mean.

<table>
<thead>
<tr>
<th>AIS Length</th>
<th>% With APP</th>
<th>% Without APP</th>
</tr>
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<tbody>
<tr>
<td>&lt; 10 µm</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>10-15 µm</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>15-20 µm</td>
<td>25</td>
<td>41</td>
</tr>
<tr>
<td>20-25 µm</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td>&gt; 25 µm</td>
<td>10</td>
<td>13</td>
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**Table V: AIS lengths for aged mice at 24-hours survival time.** Population percentages of AIS with direct APP association were higher than the AIS population with no apparent APP association in <10 µm and 10-15 µm categories and lower in 15-20 µm, 20-25 µm, and >25 µm categories. Data are presented as mean ± SEM.
Figure 3.9 Shift towards shortening in populations associated with APP in both young and aged mice at 24-hours survival time. Significant shift towards shortening in populations associated with APP in aged mice (B). Though not significant due to low n number, analogous population shift was also seen in young mouse (A). Data are presented as mean ± SEM. (* p < 0.05)
Figure 3.10 Shift towards shortening in populations at 24-hours associated with APP in young and aged mice compared to sham. Data are presented as mean ± SEM.
Figure 3.11 No significant difference between AIS in sham-injury and AIS without APP at 24-hours survival time in young and aged mice. Data are presented as mean ± SEM.
AIS Lengths were Not Significantly Different Between Young and Aged Mice

Following quantitative assessment of AIS lengths in each APP positive or negative subpopulation between young and aged mice at different survival times, corollary groups between ages were then compared. To establish whether shortening occurred concomitantly with age, independent of injury, AIS lengths were compared between young and aged sham-injured animals. Employing the same length categorization procedure to develop population profiles, no significant difference (ANOVA, \( p > .05 \)) was seen in any length category between young and aged sham-injured animals, suggesting that age alone does not play a direct role in determining AIS length (Figure 3.4 A, B; Figure 3.13).

To determine whether age played a significant role in determining length of initial segments post-injury, AIS lengths were compared between young and aged mice at each recovery time following cFPI. When AIS with direct APP association were compared between young and aged animals at 3-hours survival time, no significant difference (ANOVA, \( p > .05 \)) was detected between any of the five length categories (Figure 3.1 B, E; Figure 3.11). Similarly, at 24-hours survival time, no significant difference (ANOVA, \( p > .05 \)) was observed in APP associated AIS subpopulations between young and aged mice (Figure 3.6 B, D; Figure 3.15).
Figure 3.12 No significant difference between AIS lengths in young and aged sham-injured animals. Data are presented as mean ± SEM. (* p < 0.05)
Figure 3.13 No significant difference in AIS lengths associated with APP between young and aged mice at 3-hours survival time. Data are presented as mean ± SEM. (* $p < 0.05$)
Figure 3.14 No significant difference in AIS lengths associated with APP between young and aged mice at 24-hours survival time. Data are presented as mean ± SEM. (* $p < 0.05$)
Qualitative Assessment Suggests Progressive AIS Degradation Following cFPI is More Profound with Age

*AIS are Morphologically Altered with Age Independent of Injury*

In Layer V of the neocortex, qualitative differences between young and aged animals were apparent based on anG and APP immunoreactivity in both sham and injured groups. Following sham-injury and APP immunohistochemistry, minimal background APP staining was noted and neither age group exhibited APP immunoreactivity profiles indicative of TAI. AnkG immunoreactivity, however, displayed visible differences in the general population, as well as frequent morphological diversity in AIS populations between the young and aged animals. For example, AIS from young, sham-injured mice were consistently morphologically similar in terms of length, size, and shape, appearing generally linear and demonstrating relatively gradual curvature (Figure 3.4 A). In contrast, AIS from aged, sham-injured mice revealed AIS populations that were generally of thinner caliber and more tortuous, displaying a sharply contorted, serpentine shape (Figure 3.4 B). Both young and aged AIS populations in sham-injured mice showed clear, uniform directionality and limited ankG fragmentation evidenced by discontinuous labeling. (Figure 3.4 A, B).

*AIS Comparison of Young and Aged Animals Following cFPI*

Confocal analysis at 3-hours survival time post-injury in both young and aged animals showed an apparent increase in AIS fragmentation, a trend that was qualitatively more pronounced in aged animals as compared to young (Figure 3.16 A, C). At 24-hours
survival time post-injury, in both young and aged animals, there was clear evidence of progressive APP swelling (Figure 3.6 B, D). At this time point, qualitative differences between young and aged mice appeared more profound than either the 3-hour survival times or sham-injured mice. At 24-hours survival time, young mice showed evidence of a minor increase in total AIS fragmentation, however, cytoskeletal integrity appeared to remain intact. Also present in young mice were morphological abnormalities in AIS shape resulting in sharp contortions, similar to those seen in aged mice (Figure 3.16 B).

Though disruptive changes were evident in young animals, they appeared far more pronounced in aged animals. At 24-hours survival time, fields taken from aged mice appeared substantially more disordered with large, disjointed fragments of ankG found in seemingly random orientation (Figure 3.17 B). In addition to larger fragments with no discernable orientation, AIS displaying fracturing were also more commonly observed in populations at 24-hours than at 3-hours survival time (Figure 3.16 D). Though fracturing caused discontinuity in ankG labeling, clear connections between fragments could be drawn based on proximity and conserved orientation. It is important to note that AIS fracturing was observed in axons associated with APP, as well as axons with no apparent APP association. When viewed in temporal progression, qualitative assessment of each injury group suggests the pathobiological changes occurring in aged mice are more prominent (Figure 3.4; Figure 3.6). In extreme cases involving aged mice, evidence suggests an elevated progression of pathobiogenesis, causing complete disruption of the AIS cytoskeleton with widespread ankG dissolution (Figure 3.17 C).
Figure 3.15 AIS fracturing appears more evident in both age groups at 24 hours. Minor increase in fracturing of AIS in young mice at 3-hours (white arrows, A). More apparent increase in AIS fracturing (white arrows) at 3-hours in aged mice (C). Following this trend, fracturing was more apparent in at 24-hours compared to 3-hours. This that appeared more profound in aged (D) than young mice (B). AIS of young mice at 24-hours appeared to be more tortuous than 3-hours or sham (yellow arrows, B). Scale 20 μm.
Figure 3.16 Pronounced fragmentation of AIS appeared more profound in aged mice at 24-hours. Aged mice appeared substantially more disordered with large, disjointed fragments of ankG found in seemingly random orientation (white arrows, B). Fragmentation could be observed in young mice but appeared considerably less pronounced (white arrows, A). In the most extreme cases of aged mice, complete breakdown of the AIS evidenced by dissolution of ankG was observed (C).
DISCUSSION

The purpose of this study was to determine if diffuse TBI was associated with structural alterations to neuronal AIS length in the neocortex, and to determine whether advanced age exacerbated TBI severity. Consistent with previous diffuse TBI/DAI reports, APP swellings were observed interspersed throughout regions of the neocortex, adjacent to axons with no apparent APP swellings (Povlishock et al., 1983; Adams et al., 1989; Smith et al. 2003) and the prevalence of these swellings was greater in the aged sham injured mice as compared to the young sham injured animals. This elevated level of APP swellings, however, was exacerbated following injury. These findings confirm age as a risk factor in TBI and identify axonal injury as a potential mediator of this increased vulnerability.

Although the prevalence of APP swellings was increased with age, both in the injured and sham mice, no difference in AIS length was observed between the age groups. In both aged and young mice, the length of the AIS was significantly shortened for those segments that exhibited APP accumulations; however, no difference was observed between age groups. In addition, AIS that were not associated with APP accumulations revealed no difference in length either with regard to survival time or age.

Although the quantitative length analysis of the AISs between the age groups revealed no significant difference, qualitative analyses suggested that the AISs of aged mice are more fragmented and exhibit a more tortuous shape. Perhaps more importantly, these morphologic differences become more profound following injury. Presently, these differences have only been qualitatively identified and detailed quantitative analyses will
be required to confirm these potential pathologies. The potential significance of these observations are discussed below.

**Structural Alterations to the AIS Result in Functional Modification**

To assess AIS structural alteration, lengths of initial segments were measured and segregated into groups based on whether direct APP association could be established. Numerous studies have shown that physical properties, such as AIS length, play a crucial role in normal AIS function (Colbert and Johnston, 1996; Khaliq and Raman, 2006; Palmer and Stuart, 2006; Kole et al., 2007; Shu et al., 2007). It is currently understood that the AIS is a unique, highly specialized, axonal domain responsible for action potential (AP) initiation and maintenance of neuronal polarity (Stuart and Sakmann, 1994; Mainen et al., 1995; Buffington and Rasband, 2011). Preservation of AIS cytoskeleton plays a significant role in normal neuronal function, as loss of ankG expression has been tied to the loss of neuronal polarity (Hedstrom et al., 2008). Disruptions to the AIS cytoskeleton, evidenced in pathological alterations in AIS morphology, have been found to result in changes in neuronal excitability (Kuba, 2012), causing overall disruption to the networks in which they function (Baalman et al., 2013). The change in intrinsic neuronal excitability, in the context of AIS cytoskeletal degradation, could be associated with focal disruption of predominant voltage gated sodium channels (Na\textsubscript{v}) 1.6 and 1.2, which have been identified as functionally causal constituents for driving AP initiation and proper overall AIS function (Buffington and Rasband, 2011) and whose precise clustering in the AIS is dependent on ankG (Kordeli et al., 1995; Davis et al., 1996; Zhou et al., 1998). Na\textsubscript{v} 1.2, the CNS immature sodium
channel isoform, is found clustered in the proximal region of the AIS, directly adjacent to axon hillock and is believed to be involved in regulation of AP backpropagation to the neuronal somata and dendrites (Hu et al., 2009). Aggregates of the mature isoform, Nav 1.6, are generally found in more distal portions of the AIS and are believed to be a major contributor to AP initiation (Buffington and Rasband, 2011). Within the context of TBI, Nav 1.6 has been demonstrated to be a target of calpain-mediated proteolytic cleavage, specifically at its α-subunit (Iwata et al., 2004). Following in vitro dynamic stretch injury, a related DAI model, cleavage resulted in persistent Na, 1.6 permeability, leading to sustained pathologic influx of sodium ions (Na⁺), causing sodium channel dysfunction (Iwata et al., 2004; von Reyn et al., 2009, 2012).

Prior studies using ischemic (Hinman et al., 2013), blast (Baalman et al., 2013), and cFPI (Greer et al., 2013) models have observed similar injury-induced physical alterations to the AIS causing a decrease in length based on ankG immunolabeling of the domain, as seen in the present study. In the cFPI model, this consistent plastic response resulting in shortening was tied to a temporary, incomplete disruption of the AIS cytoskeletal-scaffolding proteins and delayed restoration of neuronal excitability (Greer, et al., 2013). Importantly, this incomplete disruption did not cause the relative stability of ankG to be compromised at any observed time point post-injury, nor was there evidence to suggest that Nav 1.6 cleavage had occurred (Greer, et al., 2013). The scope of this study, however, was limited to acute effects within 12 hours post-injury in young mice, from 8-12 weeks old.

Results from the present study pertaining to AIS integrity are consistent with the findings of Greer and colleagues when only comparing young mice groups,
approximately 8 weeks old. Although qualitative in nature, our findings revealed contrasting results in aged mice. At 24-hours survival time, ankG labeling in aged mice appeared substantially more disordered with large, disjointed fragments found with no discernable orientation, which could be an indication of cytoskeletal disruption within the AIS, a development which had not been previously observed following cFPI (Reeves et al., 2010; Greer et al., 2013). This disruption could suggest that degradation of scaffolding complexes of ankG become untethered from βIV-spectrin and represent the initial stages of ankG proteolysis via the calcium-dependent cysteine protease calpain, which has been shown to cause complete ankG breakdown following ischemic insult (Schafer et al., 2009). The conventional TAI mechanisms involving axolemmal mechanoporation leading to pathologic calcium (Ca\(^{2+}\)) influx, activation of Ca\(^{2+}\) proteases, and subsequent channelopathies are believed to have a diminished role in AIS following TAI due to the conservation of AIS stability (Greer et al., 2013). However, the evidence supporting this hypothesis is contingent upon preservation of the AIS cytoskeletal integrity, which was observed to be structurally compromised in aged mice in the present study. Though the mechanism still remains unclear, present findings suggest that ankG may be more vulnerable to mechanical or biochemical perturbations following injury as a consequence of age with the potential for more severe pathobiological consequences resulting from TAI.

**AIS Cytoskeleton is Required to Maintain Functionality**

The preservation of the AIS cytoskeleton in young mice has important implications regarding neuronal fate. Retention of the AIS cytoskeletal integrity is
essential to maintaining neuronal polarity (Hedstrom et al., 2008). Sustained neuronal polarity has been demonstrated to be a required component for continued molecular identification of the primary neurite/axon in the regenerative response of axon sprouting in the proximal axonal segment following mild TBI (Greer et al., 2011). Based on observations from the current study, the cytoskeletal retention seen in young mice at 24-hours survival time may implicate a more prompt or effective axonal regenerative response, whereas aged mice at 24-hours survival time with damaged AIS cytoskeletons may be incapable of mounting a similar response with the same efficacy. Current evidence based on ischemic models may even suggest that any neuroplastic response may not be possible if complete dissolution of the AIS occurs, causing neurons to become non-functional due to loss of neuronal polarity and AIS Na$_v$ channel clusters (Schafer et al., 2009), though this phenomenon has not been previously observed in cFPI models. It is important to note there was no evidence for injury-induced apoptosis in the ischemic model, a feature that is consistent with atrophic changes in the absence of injury-associated cell death within prior cFPI studies (Singelton et al., 2002; Lifshitz et al., 2007; Greer et al., 2011).

**APP Swellings Used to Monitor Axonal Transport and Somatic Response Following DAI**

As APP has been shown to be a consistent marker of axonal transport pathology (Gentleman et al., 1993; Greer et al., 2013), the progressive linear and radial enlargement of APP swellings from 3-hours to 24-hours could indicate disruption in normal anterograde axonal transport. However, extensive evidence has shown that once swelling
is attenuated, APP accretions become stable and a transformation occurs at the site of the proximal APP swelling resulting in a shift from further anterograde to retrograde axonal transport (Sahenk and Lasek, 1988; Martz et al., 1989; Smith and Snyder, 1991; Snyder et al., 1994; Greer et al., 2011; Greer et al., 2013). The anterograde-retrograde conversion has been associated with early reorganization and recovery of the proximal axonal segment, an early feature of the axonal regenerative response (Greer et al., 2011). If the regenerative response differed between young and aged mice, it could be evident in APP swellings measured at various survival times beyond 24-hours after insult. Research has shown that at 48 hours post-injury, the length of APP swellings in direct continuity with their sustaining somata was significantly reduced and by 72 hours most APP swellings were no longer observed (Singleton et al. 2002). Following TAI, potential differences in neuroplastic responses between young and aged mice could help establish candidate models for uncovering possible age-related mechanisms.

**Increased Fragmentation may Indicate Increased Vulnerability with Age**

In the current study, AIS fracturing was observed in axons directly associated with APP, as well as axons with no apparent APP association, which could suggest a pathobiological impact on the neuronal population not directly associated with TAI. This hypothesis is supported by prior studies observing electrophysiological abnormalities within nonaxotomized pyramidal neurons at 24-hours post-injury (Greer et al., 2012), and intense staining of the phosphorylated alpha subunit of eukaryotic translation initiation factor 2 in neuronal somata that were neither axotomized nor associated with any somatic
increase in APP in the mediadorsal neocortex at 24-hours post-injury (Singleton et al., 2002). Fracturing within the AIS could potentially be a sign of further degeneration. Based on qualitative analysis, fracturing appeared more common in aged mice, which could suggest that axonal populations not directly associated with APP might be more vulnerable in aged mice following injury causing further injury-induced neuroplastic changes.

**Could Sinuosity of AIS Impact Overall Stability?**

AIS cytoskeletal sinuosity may be structural evidence that some form of plastic response has occurred in the initial segment. Synaptic plasticity, axo-axonal synaptic remodeling, and AIS length and position shifts have been shown to occur throughout the aging process within the AIS domain (Rasband, 2010; Grubb et al., 2011; Kuba, 2011; Kuba et al., 2012). In light of prior research, this concept could aid in explaining why observations from the present study revealed AIS in aged mice to appear more sinuous, while AIS in young mice were generally linear in shape, with clear and consistent directionality.

Numerous studies have also shown that AIS plasticity also occurs following traumatic axonal injury (Greer et al., 2011; Greer et al., 2012; Hinman et al., 2013; Baalman et al., 2013). Post-injury retrograde transport is a plastic response leading to axonal reorganization proximal to the site of injury and eventually resulting in regenerative neuronal process formation, seen in the form of axon ‘sprouting’ (Greer et al., 2013). This progressive process is temporal in nature, which would explain why
evidence of tortuosity and shape modification were not evident at 3 hours in young mice post-injury but were more readily observed at 24 hours.

If these morphological variations are the result of plasticity, could they have an impact of AIS cytoskeletal stability? As additional structural modifications are made to the AIS following various plastic responses, its shape could continue to be altered over time. As these shape changes are direct reflections of physical alterations to the AIS, repeated changes could result in a compromised structural integrity of the cytoskeleton, eventually resulting in a more “fragile” AIS.

As a basic analogy: once bent, a paperclip cannot be exactly re-straightened to its original form. The stress of bending the metal adds defects to the material. Repeated bending causes defects to accumulate, causing material “fatigue”, and eventual failure in the form of breakage. Though the actual mechanisms are certainly not the same in the AIS, the concept of repeated stress causing structural fatigue may be linked. Sinuous shape could result from both age-related and injury-induced plasticity and may not be indicative of neuropathy itself; however, it could be an indication of increased biomechanical vulnerability to secondary insult, specifically to a region that is already known to show a predilection for injury.

Looking Forward

In conclusion, the current report investigates the potential for age-related consequences of diffuse TBI in the AIS of neocortical neurons. Though the AIS has previously shown a predilection for injury, the mechanisms behind such injury are poorly understood. To better evaluate pathobiological consequences specifically related to age it
would be advantageous to expand the scope of investigation for future studies. Such expansion could include incorporation of nodal/paranodal/internodal regions in which the mechanism of injury is currently better understood. As multiple swellings are known to occur along the axon, with APP demarcating the site of eventual axonal disconnection (Greer et al., 2013), a spatial approach could facilitate age-related comparisons incorporating the number, location, and size of these swellings, providing a more detailed, quantitative analysis of pathobiogenic factors related to TAI. In addition to structural comparisons, another potential area for investigation could be temporal changes related to TAI, specifically within the context of secondary progressive axotomy. Disconnection in young mice has been shown to be a rapidly progressing event, with the majority of axons demonstrating evidence of discontinuity within 30 minutes following injury (Kelley et al., 2006; Greer et al., 2013). This pathologic process could show ephemeral differences between young and aged mice that might have critical implications pathobiogenesis as well as axonal recovery and reorganization.

Based on qualitative analysis AIS cytoskeletal shape, further questions arise such as: does age-related synaptic plasticity physically alter the AIS domain structure over time? If so, could AIS alteration exacerbate pathobiological changes that qualitatively appear to be more prominent in aged mice? If age-related neuronal impairments are present, it is possible that they could be more severely amplified in aged mice following insult. Answering these questions could help in the discovery of age-related, detrimental neuroplastic responses and uncover potential candidate models for explaining differences in TAI-related injury mechanisms.
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