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The effect of repeat mild traumatic brain injury in the adolescent rat on Alzheimer's disease pathogenesis

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The effect of repeat mild traumatic brain injury in the adolescent rat on Alzheimer's disease pathogenesis

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in Neuroscience

by

Daya Alexander Grant

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ABSTRACT OF THE DISSERTATION

The effect of repeat mild traumatic brain injury in the adolescent rat

on Alzheimer's disease pathogenesis

by

Daya Alexander Grant

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2015

Professor Mayumi L. Prins, Chair

Repeat mild traumatic brain injury (RTBI) is highly prevalent among adolescents in the form of recurrent sport concussions. This epidemic is of particular concern due to its potential correlation with neurodegenerative disease. Moderate to severe TBI is a known risk factor for Alzheimer's disease (AD), but a clear relationship between RTBI and AD has not yet been established. Moreover, two critical questions are 1) does the interval between repeat injuries alter the outcome measure, and 2) are there gender differences?

Since wild-type rats do not generate ample Aβ, triple transgenic Alzheimer's rats (3xTg-AD) were required for the Aβ-related experiments. To determine a correlation between RTBI in adolescence and accelerated Aβ pathogenesis, as well as to establish the effect of a varied brain impact interval (BII), postnatal day 35 3xTg-AD rats received a sham injury, four injuries spaced 24 hours apart ($4RTBI_{24}$), or four injuries spaced 72 hours apart ($4RTBI_{72}$). Aβ burden was analyzed in the hippocampus, entorhinal cortex, and parietal cortex 10.5 months after the last injury, demonstrating an increased burden in the hippocampus after $4RTBI_{24}$. Interestingly, when BII extended to 72 hours, Aβ burden was not significantly different from sham. These findings were not different between males and females.

Two potential mechanistic links between RTBI and accelerated Aβ pathology were assessed in Chapter 2 and Chapter 3. First, traumatic axonal injury (TAI) was measured using diffusion tensor imaging (DTI) and analyzed using tract-based spatial statistics (TBSS). Fractional anisotropy decreased 1 week after $4RTBI_{24}$ in the corpus callosum and external capsule, but returned to baseline by 6 months. Second, biochemical analysis demonstrated an increase in Aβ oligomers, despite any changes in BACE1 or PS1, the cleavage enzymes required to produce the Aβ peptide.

This dissertation demonstrates a potential correlation between RTBI in adolescence and accelerated AD pathology, which might be eliminated if the BII is increased to allow for metabolic recovery between injuries.

The dissertation of Daya Alexander Grant is approved.

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INTRODUCTION

CONCUSSION DEFINITION AND EPIDEMIOLOGY

Concussion is considered one of the most complex sports medicine injuries to diagnose and manage due to a constantly evolving definition, the lack of clear neurological signs and biomarkers, and negative radiographic findings. A concussion is a type of mild traumatic brain injury (mTBI) that exists on the lower end of the mTBI severity scale and is generally shorter in duration. The word "concussion" was introduced in the 10th century and was defined as an abnormal physiological state distinct from severe brain injury (Rhazes, 1497). Over the next few centuries, the definition was based on clinical presentation until pathophysiological understanding grew in the 17th-19th centuries (McCrory and Berkovic, 2001). The term "concussion" is often, but not always, used interchangeably with mild traumatic brain injury (mTBI). The American Medical Society for Sports Medicine posits that a concussion is a subtype of mTBI, indicating that an mTBI is not always a concussion (Harmon et al, 2013).

Consensus statements have helped guide our understanding and management of concussions. The first consensus statement, released in the mid-1960's, defined concussion as "a clinical syndrome characterized by the immediate and transient posttraumatic impairment of neural function such as alteration of consciousness, disturbance of vision or equilibrium due to mechanical forces" (Congress of Neurological Surgeons, 1966). This definition has evolved in the past 50 years based on a growing

body of clinical and basic science research. Currently, the most agreed upon definition of concussion emerged from the 4th International Conference on Concussion in Sport held in Zurich, 2012. This consensus statement is commonly referred to as the "Zurich guidelines" and defines a concussion as "a complex pathophysiological process affecting the brain, induced by biomechanical forces" (McCrory et al., 2013).

Although a concussion is classified as a mild TBI (mTBI), it still produces a range of physical and cognitive symptoms. Concussion symptoms are typically classified in to four categories: physical, cognitive, emotional, and sleep (Table 1). Headache is the most common symptom and occurs in over 97% of cases (Meehan et al., 2010). Dizziness, difficulty concentrating, and confusion are also commonly reported, occurring in approximately 75%, 55%, and 46% of cases (Meehan et al., 2010; Makdissi et al., 2010; Marar et al., 2012). Other symptoms include nausea and vomiting, sensitivity to light and sound, vision problems, loss of balance, amnesia, fatigue, and changes in sleep patterns (Halstead et al., 2010). Some of these symptoms are non-specific or overlap with other disorders. For example, headache and fatigue can occur frequently in the general population and many symptoms overlap with depression, attention deficit disorder, and sleep disturbances (Harmon et al., 2013). Therefore, diagnosis is usually based on evaluation tools such as the standardized concussion assessment tool (SCAT3), which includes a symptom checklist and a cognitive and physical evaluation. Loss of consciousness is often incorrectly assumed as a criterion for concussion; however, it only occurs in 5-19.2% of concussions and is not necessary for a diagnosis (Collins et al.,

2003; Meehan et al., 2010). Overall, approximately 80-90% of concussions resolve spontaneously within 7-10 days (Macciocchi et al., 1996; McCrea et al., 2005; Makdissi et al., 2010; Meehan et al., 2010; Frommer et al., 2011; Marar et al., 2012), but up to 10 to 17% of individuals will experience prolonged symptoms.

Concussion symptoms
Physical
Headache
Dizziness
Light sensitivity
Balance problems
Blurry / double vision
Nausea
Fatigue
Loss of consciousness
Numbness / tingling
Cognitive
Difficulty concentrating
Confusion
Amnesia
Fogginess
Delayed reaction time
Feeling stunned
Emotional
Irritable
Sad
Nervous
Generally more emotional than usual
Sleep
Drowsy
Sleeping more than usual
Sleeping less than usual
Difficulty falling asleep

Table 1. Common concussion symptoms (adapted from Harmon et al., 2013)

There is a difference in the length of symptom recovery between high school and college athletes. High school athletes have longer symptom recovery than college athletes (Williams et al., 2015) and reaction time can take up to 21 days to return to baseline (Covassin et al., 2010). Furthermore, high school athletes perform significantly

worse than age-matched controls on neuropsychological testing 7 days after injury, but college athletes match control subjects by 3 days post-injury (Field et al., 2003). These cognitive symptoms can have implications beyond the athletic field with 38% of adolescent athletes experiencing difficulties re-adjusting to school after a concussion. The length of time these problems persisted was not assessed (Darling et al., 2014).

Traumatic brain injury (TBI) is highly prevalent in the adolescent population. TBI of any severity occurs 1.6-3.8 million times per year (Langlois et al., 2006) and according to the Centers for Disease Control, 75% of all TBIs are mild (CDC, 2003). 51% of total TBI occurs in people under the age of 24 (Faul et al., 2010) and 70.5% in people between 10-19 years old (CDC MMWR, 2011), which is the age range for adolescents as defined by the World Health Organization. As can be appreciated in Figure 1, the annual incidence of nonfatal sports and recreation-related TBI that present to emergency departments in the United States peaks during adolescence. The aforementioned incidence rates are likely an underestimation, however, since many concussions go undiagnosed and/or unreported (McCrea et al., 2004; Daneshvar et al., 2011).

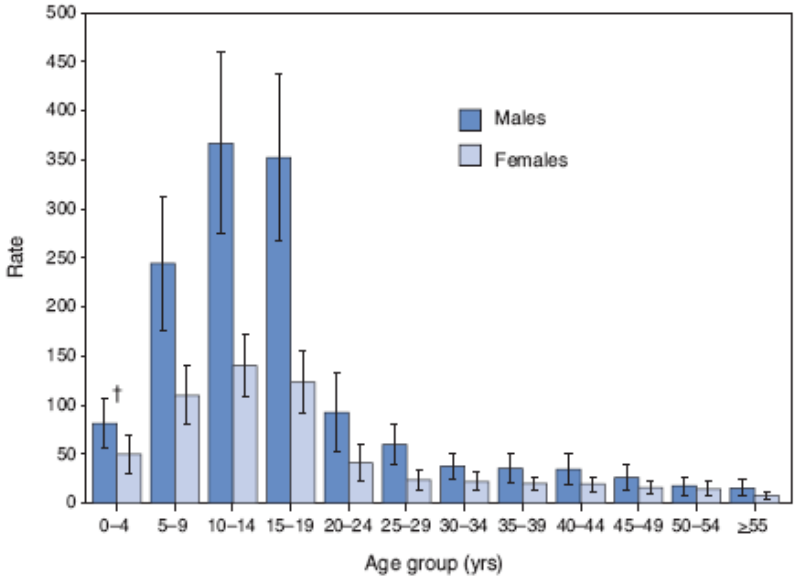

Figure 1. Annual rate (per 100,000) of nonfatal sports- and recreation-related TBI treated in emergency departments (Reprinted from MMWR, 2007)

The high rate of mTBI in adolescence is due to sports-related concussions and is sport-specific. In the context of all athletic injuries, mTBI accounts for up to 8.9% of total injuries in high school varsity sports and 5.8% of all collegiate athletic injuries (Gessel et al., 2007; Powell et al., 1999). The occurrence of concussions varies significantly between sports. To determine these rates, the number of athlete-exposures (AEs) is often determined, which is defined as one athlete's participation in one practice or competition (Table 2). In 20 high school sports, concussions were reported at a rate of 2.5 per 10,000 AEs (Marar et al., 2012). Football has the highest incidence of concussion followed by girls' soccer and boys' lacrosse, within high school and college athletics (Lincoln et al., 2011). Contrary to popular belief, concussions in soccer are mainly due

to head-to-head, head-to-body, or head-to-ground contact and not due to heading the ball, as is often assumed (Matser et al., 2004). These high overall rates of concussion in high school and college sports have increased significantly over the past decade, which may be due to more widespread awareness of TBI (Rosenthal et al., 2014) and an evolving clinical definition of concussion. It is important to note that while concussion studies have been conducted outside of the United States, the overwhelming majority focused on elite athletes who are usually older than adolescents. However, non-U.S. adolescent studies have reported high incidence rates among athletes in rugby and Australian Rules football, hockey, and soccer (Shuttleworth-Edwards et al., 2008).

	Average rate of concussions per 1000 athletic exposures (AEs)			
	Females		Males	
	High School	College	High School	College
Football			0.54	0.57
Ice Hockey		0.73	0.58	0.64
Soccer	0.32	0.60	0.21	0.41
Lacrosse	0.20	0.47	0.39	0.39
Basketball	0.19	0.43	0.11	0.28
Wrestling			0.17	0.54
Field Hockey	0.19	0.18		
Softball	0.11	0.14		
Baseball			0.06	0.08
Volleyball		0.18		
Cheerleading	0.10			
Gymnastics	0.08			

Table 2. Average rate of concussions per 1000 athletic exposures (AEs) for males and females in high school and college sports (compiled from several studies cited in the text).

Gender differences exist not only in the incidence of concussion, but also in the symptomatology. High school concussion rates are higher for girls in gender-comparable sports, such as soccer and basketball (Gessel et al., 2007; Lincoln et al., 2011; Marar et al., 2012; Rosenthal et al., 2014). Furthermore, among collegiate athletes, females experience more concussions during games than males (Covassin et al., 2003) and report more symptoms overall (Broshek et al., 2005). The types of symptoms seem to differ with males reporting more amnesia and confusion and females reporting more drowsiness and sensitivity to noise (Frommer et al., 2011). In terms of outcome post-concussion, female high school soccer players have slower reaction time post-concussion than their male counterparts (Broshek et al., 2005; Colvin et al., 2009). However, the opposite is true for college athletes (Covassin et al., 2010). Furthermore, female college athletes with 2 or more concussions performed better than males on verbal and visual memory tasks (Covassin et al., 2010). The reason behind gender differences in concussion rates and reported symptoms has not yet been established. The dichotomy may be due to a host of causes, such as physiological, muscular, neuroanatomical, cultural, or hormonal, although the effect of estrogen is either protective or detrimental depending on the study referenced (Broshek et al., 2005). High school girls have a smaller neck size and less neck strength than high school boys (Collins et al., 2014). Furthermore, basal neural metabolism differs between genders (Andreason et al., 1994), which may be exacerbated following the increased metabolic demands after injury. Another possibility, although extremely controversial, is culture. Society as a whole, in the U.S., has tended to be more protective of female athletes than male athletes (Vertinsky, 1994) and males have

demonstrated a reluctance to report a concussion, perhaps for fear of being removed from play (McCrea et al., 2004). Despite these gender differences, symptom resolution time and return-to-play time are not statistically different between males and females (Frommer et al., 2011).

While males and females perform differently on neurocognitive tests after a concussion, gender differences also exist in baseline neurocognitive tests and in concussion-like symptoms. Female college athletes perform better on baseline verbal memory tasks, but males performed better on baseline visual memory tasks. Females also reported higher mean symptom scores at baseline and higher individual scores for several symptoms such as headache, nausea, fatigue, drowsiness, sensitivity to light and noise, nervousness, difficulty concentrating, and visual problems (Covassin et al., 2006).

INCIDENCE AND EFFECT OF REPEAT TRAUMATIC BRAIN INJURY

Although the incidence of repeat TBI (RTBI) in the adolescent population is not entirely known, it is estimated that 7.7% to 34.9% of concussions in high school sports are repeat insults (Langburt et al., 2001; Collins et al., 2002; Field et al., 2003; Moser et al., 2005). This range is similar among college athletes with 5.6% to 36% of concussions being repeat insults (Collins et al., 1999; Guskiewicz et al., 2003; Field et al., 2003; Slobounov et al., 2007), but the majority of this data was acquired in football, so it may be a skewed sample.

Repeat concussions are associated with worsened cognitive symptoms (Collins et al., 1999), such as impaired attention and concentration (Moser et al., 2005). A history of 2 or more concussions among high school and college athletes is associated with slower recovery time (Guskiewicz et al., 2003; Eisenberg et al., 2013), processing speed (Gronwall et al., 1975; Collins et al., 1999), reaction time, and verbal memory (Covassin et al., 2008; Iverson et al., 2012). This previously concussed cohort also had more severe symptoms, such as longer loss of consciousness, confusion, and anterograde amnesia (Collins et al., 2002). Furthermore, two days post-injury, high school and college athletes with 3 or more concussions demonstrated significantly poorer memory performance than those with a single concussion (Iverson et al., 2004). Schatz and colleagues (2011) compared the results of baseline concussion evaluations between three groups of high school athletes: 1) no history of concussions, 2) a history of a single concussion, or 3) a history of two or more concussions. Those with two or more concussions scored higher on the symptom checklist of physical, cognitive and sleep disturbances, replicating the results of an earlier study (Covassin et al., 2010).

Conversely, some studies have failed to find an effect of RTBI on baseline symptoms (Covassin et al., 2008) or neuropsychological testing (Guskiewicz et al., 2002; Iverson et al., 2006; Bruce and Echemendia, 2009) among high school and college athletes. In a retrospective study of over 200 collegiate athletes, computerized neurocognitive test results (i.e. Headminder concussion resolution index and ImPACT) were compared between 4 groups depending on the number of past concussions reported

(zero, one, two, or three). There were no differences between groups, suggesting that concussion history is not associated with performance on computerized neurocognitive tests, but as the authors highlighted as a limitation, there were a small number of cases in the two-injury group (Broglio et al., 2006). A small number of studies have failed to show detrimental effects of RTBI, which may be due to the method by which researchers differentiated injury groups or sensitivity of the outcome tests employed. However, the majority of research in this area does demonstrate a negative relationship between RTBI, symptom number and severity, and cognitive deficits.

In addition to the worsened acute effects, repeat concussions may result in long-term cognitive and neural-level impairment. In a self-report study, retired professional football players with a history of three or more concussions reported significant issues with their memory and were clinically diagnosed with mild cognitive impairment (MCI) at a higher rate than players with no concussion history (Guskiewicz et al., 2005). These former athletes ranged from recent retirees to those who played football during World War II. In this same cohort of retired athletes, a diagnosis of depression was 1.5 times more likely to occur in those with a history of 1 or 2 concussions, and 3 times more likely in those with more than 3 previous concussions, compared with the control group (Guskiewicz et al., 2007). In a study suggesting that neural-level impairment might persist long after clinical symptoms resolved, De Beaumont and colleagues (2007) found electrophysiological changes 3 years after RTBI that were not present in the single concussion cohort. In a separate study, the researchers also found motor cortex inhibition

several years after RTBI, ranging from 2 to 5 concussions (De Beaumont et al., 2007). Furthermore, the same research group examined neural and cognitive decline in 50-65 year olds thirty years after they sustained 1 to 5 concussions. The researchers measured the brain's electrophysiological response to stimulus, particularly responses associated with attention shifting and the motor system. They found electrophysiological changes in cognitive and motor systems as well as reductions in measures of episodic memory and frontal lobe functions in the concussed athletes compared to controls. Additionally, their executive motor control was slower, indicating bradykinesia (De Beaumont et al., 2009). One limitation of human RTBI research, as is evident by the aforementioned studies, is the pooling of subjects with a range of previous concussions. Researchers rely on this method due to restricted numbers of participants. However, this method prevents the analysis of a dose-dependent or graded response to multiple injuries. Furthermore, it does not allow for the analysis of brain impact interval (BII), or the effect of injury spacing on outcome measures. Lastly, retrospectively recalling concussion history is not reliable.

It is important to note that a history of concussion increases one's risk of having a subsequent concussion (Saunders and Harbaugh, 1984; McCrory and Berkovic, 1998; Cantu et al., 1998; Guskiewicz et al., 2003) and may be associated with psychological disorders later in life. High school and college football players with a history of concussion are 3 to 5.8 times more likely to sustain a subsequent concussion than their colleagues without a concussion history (Guskiewicz et al., 2000; Zemper et al., 2003). In terms of a psychological effect, there was a linear relationship between history of

concussions and a diagnosis of depression later in life (Guskiewicz et al., 2007; Kerr et al., 2012; Didehbani et al., 2013). In conclusion, repeat concussions in adolescents may have profound impacts on the severity and duration of symptoms, impaired performance on neurocognitive testing, and potentially psychological issues later in life.

RETURN-TO-PLAY & INJURY INTERVAL

The amount of time an athlete should stay out of his or her sport before being allowed to return-to-play (RTP) is one of the critical issues regarding adolescent concussion. There is a period of vulnerability after a concussion during which the brain is more susceptible to sustaining another concussion (Guskiewicz et al., 2003). If a second concussion occurs during this vulnerable period, the subsequent symptoms are greater in magnitude and duration (Vagnozzi et al., 2008). The main evidence-based concussion guidelines are from the 4th International Conference on Concussion in Sport held in Zurich (McCrory et al., 2013). It is comprised of a graduated step-by-step approach (Table 3), making the process dependent on the individual athletes instead of reliant on a set schedule. The RTP guidelines are often used in conjunction with a concussion assessment tool, such as the sports concussion assessment tool (SCAT), SCAT3, or the children's SCAT (ChildSCAT), depending on the athlete's age. However, these tools are intended for use in diagnosing concussion (McCrory et al., 2013) with sensitivity and specificity diminishing rapidly with increases in the time post-injury. In a study evaluating the effectiveness of the Zurich guidelines in adolescents, 100% of the

athletes assessed successfully returned to their sport. Interestingly, and in alignment with the personalized graduated RTP guidelines, the length of time before which an athlete could RTP varied greatly (Darling et al., 2014). One difficulty in implementing these guidelines is that the current sport culture does not always encourage the reporting of concussions, so there is often a lack of compliance with these RTP guidelines, which puts athletes at risk (McCrea et al., 2004; Yard and Comstock, 2009).

Stage	**Functional exercise component**	**Objective**
1. No activity	Rest only (physical and cognitive)	Recover
2. Light aerobic exercise	Keep heart rate (HR) <70% maximum (No resistance - walking, swimming, stationary cycling OK)	Slowly increase HR
3. Sport-specific exercise	Simple drills without head impact	Add movement
4. Non-contact training drills	More complex drills	Increase coordination and cognitive load
5. Full contact practice	Normal training/practice participation	Assessment of functional skills and improved confidence
6. Return to play	Resume full game play	

Table 3. Graduated return-to-play protocol (adapted from McCrory et al., 2013)

Although the Zurich guidelines reflect a greater understanding of concussion, they still contain some limitations. First, the symptoms section of the guidelines lacks specificity, since all of the concussion symptoms overlap with and can be attributed to

non-concussion disorders in the general population. Second, as the guidelines mention, complete cognitive and physical rest may have limits, beyond which further rest can be detrimental to one's psychology during recovery. Lastly, the graduated protocol requires the athlete to be asymptomatic before he or she returns to play. This is problematic due to the potential for uninjured people to present with concussion-related symptoms. However, the guidelines do acknowledge that "low-level exercise for those who are slow to recover may be of benefit" (McCrory et al., 2013). More research needs to be conducted to provide evidence-based justification for the next iteration of concussion diagnosis and management guidelines.

The findings in human studies that serve as the basis for return-to-play guidelines have been replicated in animal studies, where variables can be more tightly controlled. Some studies support the theory of a temporal window of metabolic brain vulnerability after a concussion. Prins and colleagues (2013) used ^{14}C-2-deoxy-D-glucose (2DG) autoradiography to measure the cerebral metabolic rate of glucose (CMRglc) after a closed-head single or repeat mTBI in postnatal day 35 rats. Repeat injuries were spaced either 24 or 120 hours apart to study the metabolic effects of brain impact interval (BII). After the single mTBI, CMRglc decreased the parietal cortex and hippocampus at 24 hours, but then returned to sham levels by 3 days. This metabolic recovery profile was similar after the second injury when the BII was 120 hours. However, when the BII was 24 hours, the second injury resulted in CMRglc depression greater in magnitude and duration. Similarly, mTBI repeated at varying intervals (e.g. 1, 2, 3, 4, and 5 days)

causes differential changes in brain metabolites (Vagnozzi et al., 2007) and reactive oxygen species in adult rats (Tavazzi et al., 2007). Specifically, cerebral metabolic function (as measured by N-acetylaspartate [NAA] and N-acetylglutamate [NAG]) was the most impaired with a BII of 3 days. Injured animals only performed at sham levels when injuries were spaced 5 days apart (Vagnozzi et al., 2007). The same pattern was observed with reactive oxygen species (Tavazzi et al., 2007). Clearly, BII is an important area of study, considering the concern for when an athlete is safe to return to play.

Animal studies have also examined the effects of BII on behavioral outcomes. Rats subjected to repeat injuries spaced 3-5 days apart experienced greater cognitive deficits in spatial learning and memory tasks at acute time points than when the injury interval is spaced 7 or 20 days apart (Longhi et al., 2005; Weil et al., 2014). These deficits persist chronically, with performance in the Morris water maze 6 months after injury (in 3-month old mice) significantly worse when BII was 24 hours or 1 week compared to sham (Mannix et al., 2013). However, when BII was increased to 2 weeks or 1 month, there was no statistical difference in chronic performance between sham and injured (Meehan et al., 2012; Mannix et al., 2013). Overall, increased BII is positively correlated with improved behavioral outcomes following RTBI.

IMAGING CONCUSSION

Diffusion Tensor Imaging

Researchers have begun using advanced neuroimaging techniques to assess white matter microstructure after TBI. Currently, a diagnosis of concussion is based on self-reported symptoms, since concussions cannot be imaged by standard techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT). This is problematic because athletes may fail to report their symptoms (Dziemianowicz et al., 2012) either because they cannot articulate their symptoms or, as is more often the case, because they want to continue playing (Chrisman et al., 2013). Diffusion tensor imaging (DTI) was first introduced in the mid-1990s as a non-invasive, advanced MRI technique (Basser et al., 1994). Recent research suggests that DTI has the potential to be a biomarker for mTBI due to its sensitivity to detect axonal injury, a feature lacking in other imaging modalities, such as structural MRI or CT (Rugg-Gunn et al., 2001; Arfanakis et al., 2002; Inglese et al., 2005). DTI measures the diffusivity of water molecules, which predominantly travel parallel to healthy, heavily myelinated white matter tracts. However, when axons are disrupted (e.g. following axonal injury), anisotropy, or the extent of directed orientation, decreases as water diffuses in multiple directions. Based on this principle, the four outcome measures most often used in TBI research are: 1) fractional anisotropy (FA), 2) axial diffusivity (AD), 3) radial diffusivity (RD), and 4) mean diffusivity (MD). FA is the degree of anisotropy in a single voxel and ranges from

0 to 1, with 0 being completely isotropic (moving equally in all directions) and 1 being completely anisotropic. It is calculated from the following equation:

$$FA = \sqrt{\frac{3}{2}} \frac{\sqrt{(\lambda_1 - \hat{\lambda})^2 + (\lambda_2 - \hat{\lambda})^2 + (\lambda_3 - \hat{\lambda})^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}$$

AD measures the principal direction of diffusion, or diffusion parallel to the axon, and is referred to as λ_1. RD averages diffusion along the other two eigenvectors (λ_2 and λ_3). MD computes a global value based on the average diffusivity in all 3 directions ($\hat{\lambda}$). These outcome measures are used to describe the microstructure of axon bundles (Figure 2), with insight in to the underlying meaning presented later in this section.

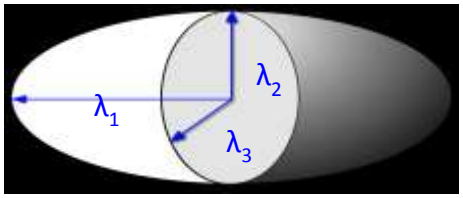

Figure 2. Representative diagram of the three eigenvectors ((λ1, λ2, λ3) used to compute DTI indices

There are changes in white matter across the lifespan of an individual. White matter volume undergoes rapid development during childhood that continues, but at a slower rate, in adolescence. It peaks at approximately 40 years of age and decreases slightly during adulthood (McLaughlin et al., 2007; Hasan et al., 2008; Lebel et al., 2012). FA also increases during childhood and adolescence before decreasing at a slower

rate in adulthood, likely reflective of neuroanatomical changes in fiber bundles. The opposite pattern is true for MD, which decreases through adolescence before gradually increasing in adulthood (Bendlin et al., 2010; Lebel et al., 2012). The time profile of these diffusion characteristics varies depending on the individual tract involved. For example, the fornix, cingulum, corpus callosum, and inferior longitudinal fasciculus develop early in childhood and adolescence, in alignment with the earlier development of basic functions such as memory, interhemispheric communication, and vision, respectively. In contrast, the frontotemporal tracts, such as the cingulum, uncinate fasciculus, and superior longitudinal fasciculus, mature last during childhood and adolescence (Lebel et al., 2012). Interestingly, there is a gender effect with white matter volume increasing at a steeper rate in adolescent males as compared to females (Paus, 2010), which is likely due to differences in testosterone concentrations between adolescent females and males (Hervé et al., 2009).

There are currently 3 main analysis techniques for DTI data, with the lack of consensus due to the rapidly evolving nature of the imaging field. As data is being acquired and analyzed, new techniques are emerging. Some studies use region-of-interest (ROI) analysis, in which the user defines areas of interest. However, this selection process introduces bias for two reasons: 1) not every region of the brain can be analyzed, and 2) it is difficult to delineate along anatomical boundaries, since DTI spatial resolution is relatively poor. Voxel-based morphometry (VBM; Ashburner and Friston, 2000; Good et al., 2001) is another analysis technique that aligns subject tensor maps

(which are used to represent the relationship between geometric vectors) to a common space and computes statistical comparisons between each voxel throughout the brain. It assumes perfect registration, which is not necessarily accomplished, and has a high chance of errors due to multiple statistical comparisons (Loring, 2002). A new and widely used methodology, tract-based spatial statistics (TBSS), has been introduced due to the limitations of the ROI and VBM analysis techniques (Snook et al., 2007). TBSS analyzes the whole brain and ensures more accurate alignment between subjects and the target space (Smith et al., 2006). The protocol for TBSS processing will be presented in Chapter 2.

DTI after mTBI

MTBI causes traumatic axonal injury (TAI), which may be detected by DTI. Post-injury, electron microscopy has revealed axonal degeneration interspersed with intact axons in the corpus callosum (Mierzwa et al., 2015). This dispersal demonstrates that axonal injury after mTBI is not diffuse, as it does not spread evenly across axonal bundles. Rather, it is multifocal, affecting degeneration in several places throughout fiber tracts. TAI results from the rapid acceleration and deceleration forces during TBI (Marmarou et al., 2005; Reeves et al., 2005; Kelley et al., 2007), which can lead to cytoskeletal perturbations, organelle accumulation, and axonal swelling (Arfanakis et al., 2002).

DTI has demonstrated time-dependent changes in white matter in both adult and adolescent brains after a single mTBI. The effect of a single mTBI on white matter in the adult human brain appears to be time-dependent, with FA increasing in the acute phase (<2 weeks post-injury) and decreasing in the chronic phase (>2 weeks post-injury; Eierud et al., 2014). The human adolescent literature follows a similar trend acutely, with FA increasing and MD/RD decreasing between 1 week and 2 months after mTBI (Wilde et al., 2008; Chu et al., 2010; Bazarian et al., 2012; Virji-Babul et al., 2013). However, two studies contrast this data, with one indicating an increase in MD (Cubon et al., 2011) and another showing no evidence of white matter abnormalities in the internal capsule or corpus callosum 72 hours, 14 days or 30 days post-concussion (Maugans et al., 2012). This conflicting data may be due to heterogeneity in the subject pool, with many studies failing to report concussion history or grouping subjects with varying numbers of previous concussions.

While studies have been performed evaluating DTI both acutely and chronically after a single concussion, very few studies have addressed the acute and chronic effects of RTBI in the adolescent brain. One study did compare a group of adolescent and young adult football players (mean age=22.5 years) who had a history of at least one concussion with age-matched controls. Since concussion history was not reported, some subjects potentially had a history of repeat concussions. The study found that FA increased in the 1-6 days post-concussion and remained elevated for six months (Henry et al., 2011). The impact of RTBI on white matter tracks has also been demonstrated in boxers who show a

substantial reduction in the number of corpus callosum fibers compared to age-matched controls (Zhang et al., 2006). There is clearly a dearth of clinical literature on the effects of RTBI, both acutely and chronically, in the adolescent brain.

Animal studies, which allow for control over the number of injuries and have been conducted to visualize the effect of TBI on white matter integrity, have attempted to rectify the conflicting results within clinical studies. However, these studies have only been conducted on the adult animal brain and primarily employ more moderate to severe injury models (Mac Donald et al., 2007; Xu et al., 2012). The limited adult RTBI studies show increased RD in both the cortex and corpus callosum during the acute and subacute post-injury phases. TBI might cause axonal breakage, which would decrease diffusion along the axon (λ_1) and potentially increase transverse diffusion (λ_3 and λ_3). After initial breakage or misalignment, axonal transport is disrupted and organelles and proteins accumulate, causing local axonal swelling (Arfanakis et al., 2002). At a more chronic time point, 60 days after experimental mTBI, RD increases in the anterior corpus callosum (Donovan et al., 2014). In one RTBI study, FA was not significantly different between 3-month old injured and sham mice 6 months after injury (Mannix et al., 2013). With the exception of these latter two studies, most studies utilize a moderate or severe injury model, so the results are not likely to be seen, or at least seen to a much lesser extent, after milder concussions.

Researchers have attempted to provide explanations for what the changes in tensor indices after TBI actually mean. Acutely after a moderate to severe cortical

contusion injury, both FA and AD decreased, corresponding to axonal injury. One to four weeks post-injury, FA remained depressed, AD returned to baseline, and RD increased, corresponding to demyelination, edema, and axonal injury, respectively (Mac Donald et al., 2007). In another study, Budde and colleagues (2011) elegantly co-registered DTI and immunohistochemistry data by applying a 2D Fourier transform to stained animal brain sections to visualize FA on injured tissue. This method revealed that an increase in FA correlates with gliosis, while a decrease in FA corresponds to axonal degeneration. The underlying explanation may vary with time post-injury. In the early acute phase, axonal injury is present on histology and AD decreases. Gliosis takes place in the late acute phase with no corresponding DTI changes. In the subacute phase, one week to one month post-TBI, histology reveals demyelination, edema, and macrophage activation accompanied by RD. It is unknown how these indices might change after repeat injuries.

TBI AND ALZHEIMER'S DISEASE

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of dementia affecting more than 30 million people worldwide. AD was first described by Alois Alzheimer in 1906 (Alzheimer, 1906). As is the case with all neurodegenerative diseases, AD progresses along a continuum and can be divided in to

three phases. The early or preclinical phase can begin 20 years before the onset of clinical symptoms. The middle phase is often referred to as mild cognitive impairment (MCI) due to AD and reflects obvious deterioration. Early symptoms include difficulty remembering new information, such as names. It can sometimes be difficult to separate this AD-related forgetfulness from the normal effects of aging during this stage. The late phase is completely debilitating, with patients requiring around-the-clock care. By this time, people have lost most of their memory, are unable to recognize loved ones, and have increased difficulty communicating and performing daily tasks.

AD can be characterized in two ways, is marked by two main pathological abnormalities, and is related to several risk factors. The two types of AD are familial and sporadic. Familial, or early-onset, AD is caused by mutations in the APP gene and the two presenilin genes, PSEN1 and PSEN2. Sporadic, or late-onset, AD has many risk factors associated with it including the APOEε4 allele. AD is marked by the following characteristic brain abnormalities: extracellular amyloid beta plaques and intracellular neurofibrillarly tau tangles (Selkoe et al., 2001). Historically, studies have been based on the amyloid cascade hypothesis (Hardy and Higgins, 1992), which posits that amyloid beta is the driver of the disease. Other potential risk factors include diabetes, obesity, blood pressure, metabolic syndrome, smoking, and TBI. Protective factors include a healthy diet, physical activity, and intellectual stimulation (Reitz et al., 2011). The molecular mechanisms involved with amyloid beta formation will be discussed below.

The association between TBI and AD

TBI is an epigenetic risk factor for the development of Alzheimer's disease (AD; Guo et al., 2000; Plassman et al., 2000; Nemetz et al., 1999; O'Meara et al., 1997; Salib et al., 1997; Schofield et al., 1997; Molgaard et al., 1990; Mortimer et al., 1991; Graves et al., 1990; Mortimer et al., 1985). A meta-analysis showing the association between TBI and AD has an odds ratio of 1.58 (Fleminger et al., 2003). Interestingly, while women represent two-thirds of all Alzheimer's patients (Hebert et al., 2013), the association between TBI and AD is only present in males and not in females (Fleminger et al., 2003; Salib et al., 1997; Mortimer et al., 1985). This lack of correlation in females may be due to the potential neuroprotective effect of estrogen and progesterone (Simpkins et al., 2009).

In addition to epidemiological evidence, there are several pathological features that are common amongst AD and TBI patients. Amyloid beta (Aß) seems to be the driving force behind AD, with increases in Aβ peptide occurring before the accumulation of tau tangles (Naslund et al., 2000). The mechanistic underpinnings of the TBI-AD relationship have yet to be elucidated, but several possibilities exist, including cell death and chronic inflammation. However, the pathophysiology of Aβ has been the most studied, stemming from research showing Aβ in brain tissue acutely after a severe brain injury (Ikonomovic et al., 2004; Roberts et al., 1994; Roberts et al., 1991). TBI accelerates the formation of Aβ plaques and neurofibrillary tau tangles (Gavett et al., 2010), two hallmarks of AD. Aβ peptides are generated by amyloid precursor protein

(APP) cleavage, which is caused by presenilin-1 (PS1), the catalytic domain of γ-secretase, and β-secretase, beta-site APP cleaving enzyme (BACE; Selkoe & Wolfe, 2000).

The pathological hallmarks of AD are seen in the brain tissue of people who sustained a severe TBI, but the time profile of plaque development seems to vary between the AD population and those with AD and a history of TBI. Aβ, APP, and neurofilament (NF) proteins accumulate in axons within hours or days of TBI-induced death (Uryu et al., 2007; Roberts et al., 1994; Roberts et al., 1991). This rapid development of Aβ after TBI is in contrast to the pathological progression seen in AD whereby plaques appear late in the disease process and gradually increasing over time. Interestingly, plaque formation does not increase linearly over time after a TBI. Instead, plaques disappear in the months following injury (Chen et al., 2009), but are greater in number several years after injury compared with age-matched controls (Johnson et al., 2012). The diffuse plaque morphology is similar to that observed in the early stages of AD as compared to the dense core plaques seen in later stages of the disease (Serrano-Pozo et al., 2011).

In contrast to observations in moderate to severe injuries, the evidence linking mTBI to AD is largely lacking, with epidemiological studies using only clinical diagnoses instead of pathological verification. However, some recent studies found that college football players (Guskiewicz et al., 2003) and retired professional football players

(Guskiewicz et al., 2005; Guskiewicz et al., 2007) reported more TBIs and had elevated incidences of memory impairment and depression than age-matched controls.

The lack of consensus within the human literature has led researchers to use animal models to study the mechanisms underlying the relationship between TBI and AD. APP becomes immunoreactive in the acute and subacute phases after moderate TBI (Pierce et al., 1996; Lewén et al., 1996). Long-term accumulation of both APP and Aβ are seen in axonal bulbs after fluid percussion injury in adult rats (Iwata et al., 2002). However, Aβ plaques are often not seen in these experiments because non-Tg rodents have low endogenous Aβ and therefore less substrate that can aggregate (Pierce et al., 1996). It was for this reason that transgenic (Tg) AD rodent models were established to examine the TBI-AD link. Since these animals develop AD pathology 100% of the time, they cannot be used to assess the causal effect of TBI on AD. However, they can be used to determine whether TBI accelerates AD pathogenesis, such as the development of Aβ and tau deposits, and the mechanisms underlying that process. Tran and colleagues (2011) showed that Tg mice had accelerated tauopathy and increased intra-axonal Aβ deposits in the fimbria/fornix and, to a lesser extent, in the ipsilateral hippocampus, corpus callosum, and external capsule 24 hours after a severe CCI injury. Tajiri and colleagues (2013) also showed approximately 3-fold greater extracellular Aβ deposits in the cortex and hippocampus of APP/PS1 transgenic mice six weeks after moderate CCI.

While these initial studies address moderate and severe injuries in Tg animal models, there is currently only one experimental study that has evaluated the effect of

repetitive mild injuries on AD (Uryu et al., 2002). Aβ was measured 2 days, 9 weeks, and 16 weeks after either RTBI (two injuries spaced 24 hours apart) or sham in adult transgenic mice. The injury model was mild with a closed-head, but exposed skull. There was no effect of injury on Aβ burden at either the 2-day or 9 week time points. However, at 16 weeks, Aβ burden was approximately 8-times greater after RTBI than after a single TBI across all regions of interest (including hippocampus, corpus callosum, and cortex). This study demonstrated the significantly accelerating effect of RTBI on amyloidogenesis. There are currently no studies addressing the risks of neurodegenerative effects following RTBI in the adolescent brain. It is difficult to obtain strong evidence for a causal relationship between TBI and AD, but there may be support for a positive correlation between RTBI and the acceleration of AD pathogenesis, for those already predisposed.

AD-RELATED PROTEINS ACUTELY AFTER TBI

The mystery within the TBI-AD relationship lies in the lag time between the TBI and the onset of AD. To understand the underlying mechanism, researchers need to evaluate acute post-traumatic changes in AD-related proteins. Since accumulation of Aβ is known to play a role in the pathogenesis of Alzheimer's disease, induction of the amyloidogenic cascade after injury may help explain a correlation between TBI and AD. Aβ is produced in a multi-step process by proteolytic cleavage of the transmembrane β-amyloid precursor protein (APP) by two secretases sequentially, β-secretase, referred to

as β-site APP cleavage enzyme 1 (BACE1; Vassar et al., 1999) and γ-secretase, whose catalytic subunit is presinilin-1 (PS1). β-secretase forms the N-terminus of Aβ and is ubiquitous in neurons (Zhao et al., 1996). γ-secretase cleaves APP at multiple points, resulting in the formation of Aβ of various amino acid lengths: $A\beta_{38\text{-}43}$ (Selkoe & Wolfe, 2007). This sequential cleaving is necessary to release Aβ from APP and thus initiates the amyloidogenic pathway. The alternate, non-amyloidogenic pathway is mediated by α-secretase, which cleaves APP in the transmembrane region within the Aβ domain, thus preventing the intact release of Aβ (Esch et al., 1990; Figure 3).

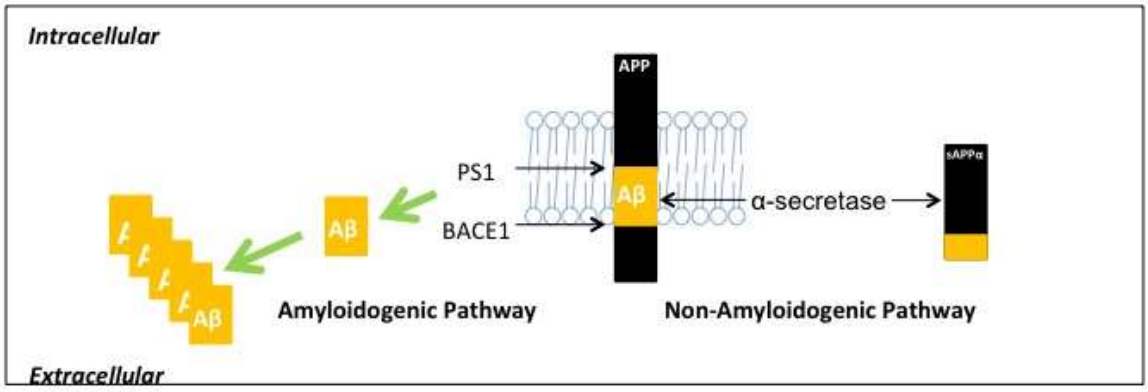

Figure 3. Cleavage of APP by BACE1 the non- and PS1 to form Aβ oligomers and plaques along the amyloidogenic pathway. α-secretase cleaves APP in the middle of the Aβ domain, initiating amyloidogenic pathway.

The amyloid cascade hypothesis (ACH) has been the predominant theory in the AD literature. However, a growing body of evidence does not support the details within the amyloid cascade hypothesis. Specifically, the number of Aβ plaques correlates poorly with cognitive function in Alzheimer's patients (Terry et al., 1991; Giannakopoulos et al., 2003). Instead, the driving force behind the disease may be

soluble Aβ oligomers (Lue et al., 1999; Mc Donald et al., 2010). In a study of AD brain tissue, levels of soluble Aβ, but not insoluble Aβ, correlated strongly with age at the time of death (McLean et al., 1999). Soluble Aβ is an umbrella term used to describe any conformation of Aβ that does not aggregate in aqueous solution after high-speed centrifugation. These oligomers may cause synaptic dysfunction, neurodegeneration, and cognitive decline (Benilova et al., 2012).

While the amyloid cascade hypothesis has yet to be completely debunked, a new wave of data is bolstering the idea that Aβ oligomers, instead of fibril-containing plaques, are the more toxic species. Traditionally, researchers thought that Aβ oligomers were simply intermediates between Aβ monomers and the aggregated fibrils. However, these oligomers may have a larger role. In an innovative study, soluble Aβ was extracted from AD patients and injected in to the mouse hippocampus. This resulted in significant inhibition of LTP and impaired memory of a learned behavior, as was measured using a step-through passive avoidance task (Shankar et al., 2008).

Given the potential importance of Aß oligomer production, temporal profiles of BACE1, PS1 and Aβ have been assessed after TBI (Figure 4). In humans, there is diffuse axonal injury (DAI) and an accumulation of BACE1 and PS1 up to 5 weeks after TBI (Uryu et al., 2007). In adult mice, BACE1 concentration in the cortex and hippocampus peaks 3 days post-CCI, but returns to baseline by 7 days (Blasko et al., 2004; Loane et al., 2009; Washington et al., 2014). This pattern is matched in slightly younger, 7 week old mice, with BACE1 levels peaking at 3 days following CCI in both the hippocampus and

corpus callosum (Yu et al., 2012). In the pig rotational, model, however, there is much more long-term accumulation of PS1 and BACE1 up to 6 months following injury (Chen et al., 2004). After a closed-head modified weight-drop injury in adult mice, PS1 increased by 2 days and peaked from 4 to 7 days before gradually declining (Nadler et al., 2008).

Since the enzymes required for Aβ production are elevated after TBI, it stands to reason that Aβ itself would increase as well (Figure 4). After CCI in adult transgenic mice, soluble Aβ40 and Aβ42 increase in both the hippocampus and the cortex from 2 hours to 48 hours post-TBI (Smith et al., 1998; Mannix et al., 2010; Tran et al., 2011; Washington et al., 2014) before returning to baseline by 72 hours post-injury (Washington et al., 2014). One study showed a slightly extended time line, with Aβ40 increasing from 24 hours to 3 days post-TBI before returning to baseline by 7 days (Loane et al., 2009). Two months after the injury, there was no difference in plaque formation (Smith et al., 1998). Two studies found a temporal difference between the two main Aβ forms, Aβ40 and Aβ42, but in contrasting directions. In an Abrahamson and colleagues paper (2006), Aβ40 decreased to sham levels 72 hours after CCI, but Aβ42, the more toxic of the species, remained elevated to the last time point of 3 weeks post-TBI. Conversely, Aβ40 decreased compared to sham 7 days after CCI in a Tg mouse, but Aβ42 did not differ between injury groups (Murai et al., 1998). The aforementioned studies all used a moderate to severe CCI model, which does not reflect the biomechanics of a concussion. A few studies have used a milder closed-head injury that showed a

delay in total Aβ accumulation, which was not statistically significant one week after TBI, but increased significantly by 4 weeks post-TBI (Laskowitz et al., 2010). Two studies measured soluble Aβ chronically post-RTBI (Mouzon et al., 2014; Mannix et al., 2013). Mouzon and colleagues (2014) used a modified CCI to deliver 5 injuries spaced 48 hours apart to adult, male mice. Neuroinflammation persisted for 12 months post-RTBI, but soluble Aβ was not elevated in the cortex or hippocampus. These results were also seen in a weight-drop RTBI model in 3-month old mice. There was no difference in soluble Aβ42 between RTBI (7 injuries in 9 days) and sham 6 months after the last injury (Mannix et al., 2013). These studies have not been replicated in adolescent animal models. It is difficult to conduct these experiments in humans, since they can only be done post-mortem. However, two studies assessed brain tissue after severe TBI and found higher levels of Aβ42, the more toxic form of the species, but not Aβ40 (Gentleman et al., 1997; DeKosky et al., 2007). The correlation between these acute, temporal changes in secretases and soluble Aβ and long-term Aβ plaque formation have yet to be determined, especially when repeat injuries occur in adolescence.

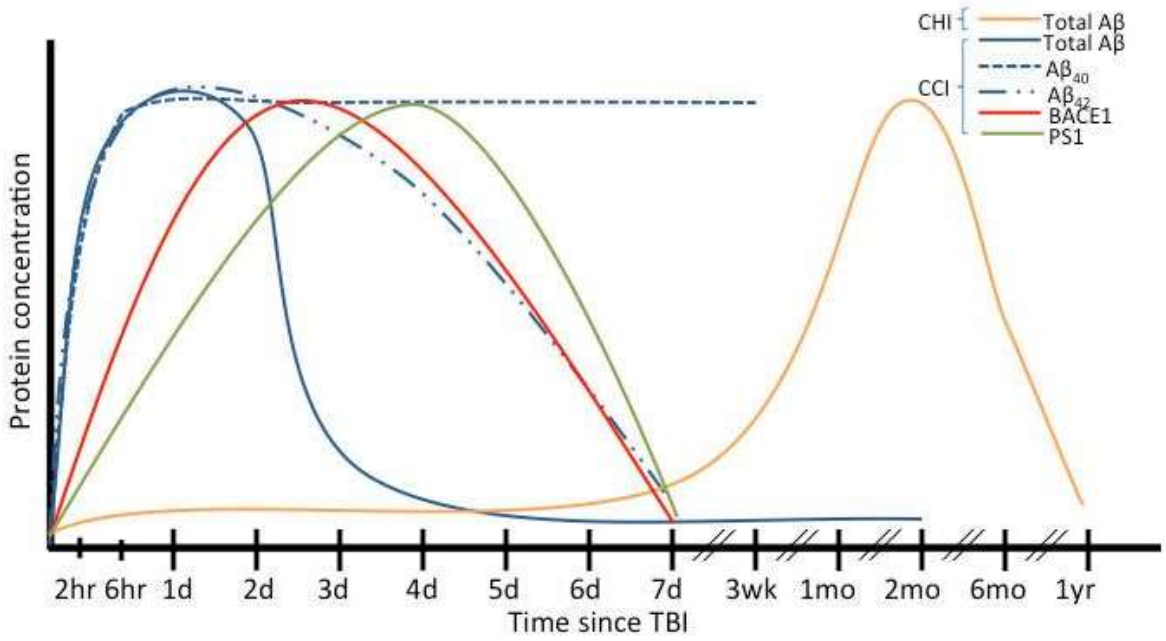

Figure 4. Schematic representation of summarized temporal profiles of Aβ, BACE1, and PS1 concentration after TBI.

EXPERIMENTAL MODELS OF TBI

Several experimental rat models of TBI exist and are used to evaluate the effect of TBI on behavior, biochemical changes and pathology. The earliest experimental concussion models were established in the early 1940's and served as the basis for physiological studies (Denny-Brown and Russell, 1940; Denny-Brown and Russell, 1941; Walker et al., 1944). Currently, the most utilized TBI models are weight-drop (Marmarou et al., 1994), lateral fluid percussion (Dixon et al., 1987), and controlled cortical impact (CCI). In the weight-drop model, a weight is released on to the exposed skull and the injury severity depends on the amount of weight and the height at which the weight is dropped (Marmarou et al., 1994). MTBI is feasible with this model, but it

results in brain stem damage due to the axis of the rat's head in relation to its body. This characteristic is uncommon with mild human injuries. The lateral fluid percussion model uses a pendulum that when released produces a wave force in to a saline-filled tube. The saline is rapidly pushed in to the epidural cavity of the brain (Dixon et al., 1987). While mild injuries can be produced, it is often difficult to control for the severity and the majority of these injuries are moderate to severe. The final of the three common models is the controlled cortical impact (CCI). In CCI, a pneumatic piston delivers a precise impact to the exposed brain or skull. All of these models usually produce moderate to severe injuries and require a craniotomy, a feature not present in human mTBI. These injuries also produce overt structural neuropathology and occasional mortality. Since human sport-related concussions are closed-head injuries that do not cause gross structural abnormalities or mortality, an accurate rodent model of concussion should not cause these characteristics.

Experimental RTBI models have also been developed for different ages. The majority of these models were characterized in the adult brain. Three closed-skull weight drop models exist for the adult mouse (DeFord et al., 2002; Creeley et al., 2004) and the adult rat (Allen, 2000). These injuries do not produce any overt pathology, but animals do display deficits on motor tasks and behavioral tasks, such as the Morris water maze. Two RTBI fluid percussion models exist for the adult rabbit (Olsson et al., 1971) and rat (Kanayama et al., 1996). Another rabbit model was established in the 1970's for the adult rat, but details of the mechanism were unclear (Weitbrecht and Noetzel, 1976). For

neonatal piglets, two repeat rotational models produce axonal injury, cognitive deficits, overt pathology, and mortality (Raghupathi et al., 2004; Friess et al., 2009). The CCI model can be modified for use on the closed head, as has been done in several adult mouse studies (Laurer et al., 2001; Uryu et al., 2002; Yoshiyama et al., 2005). These closed-head repeat injuries with CCI produce motor and cognitive deficits as well as axonal injury, as is indicated by amyloid precursor protein immunohistochemistry. Most of these aforementioned models are closed-head, which more accurately mimics human concussion, but were characterized for either the neonatal or the adult brain and not the adolescent brain.

The closed-head CCI model used in these experiments was developed to better mimic a repeat, sports-related injury in the adolescent rat. Rats are more ideal for imaging studies due to their larger brains, as compared to mice, especially for imaging with poor spatial resolution, such as diffusion tensor imaging (DTI). Consistent with human sports-related mild concussions, this injury model does not cause skull fractures, subdural hematomas or mortality. However, it does result in brief apnea as well as delayed toe-pinch response and righting time (Prins et al., 2010). This injury does not produce measurable cell loss, but when repeat injuries are spaced 24-hours apart, there is axonal damage in the white matter under the impact site one day after the second injury. At the same time point after RTBI, there is more GFAP staining around the injury site and the morphology of the astrocytes is different compared to sham. Behaviorally, RTBI rats perform worse on the Novel Object Recognition task (NOR), a measure of working

memory. For this measure, rats were exposed to two identical objects in a familiarization phase. At one day or three days post-familiarization, one of the objects was replaced with a new object. Rats are inherently curious and will spend more time with the new object. At the one-day time point, all rats performed similarly. However, three days after familiarization, single TBI and RTBI rats spent an equal amount of time with the new and the old object, indicating memory deficits (Prins et al., 2010). The rate of cerebral metabolic glucose (CMRglc) decreased 19% 24 hours after a single mTBI with this model. However, the magnitude and duration of this CMRglc depression was exacerbated after RTBI with a 24-hour brain impact interval (Prins et al., 2013). RTBI in this model also causes dysfunction of the pituitary gland, resulting in reductions of growth hormone (GH) and insulin-like growth hormone-1 (IGF-1; Greco et al., 2013).

Although human age cannot be precisely matched with rat age, based on synaptic changes (Counotte et al., 2010), tendency for high-risk behavior, and the onset of sexual maturation (Reger et al., 2009), human adolescence is best approximated by postnatal day (P) 32-45 rats. Therefore, the closed-head model used here was specifically designed for these adolescent rats.

EXPERIMENTAL MODELS OF AD

Rodents do not naturally produce substantial amounts of amyloid beta (Aβ), therefore, transgenic models of Alzheimer's disease have been developed (Pierce et al.,

1996). The vast majority of these experimental models are in mice and are based on the human genes implicated in familiar Alzheimer's disease, as compared to the much more common sporadic Alzheimer's disease. By using these genes, researchers can recapitulate the pathophysiology of AD. Transgenic mouse models can be divided in to three categories, depending on the number of mutated proteins involved: single transgenic, double transgenic, or triple transgenic. Single transgenics have APP mutations and generally develop Aβ deposits at 6-12 months (Games et al., 1995; Hsiao et al., 1996; Strurchler-Pierrat et al., 1997; Buttini et al., 2002; Richardson et al., 2003). Aβ is first detected in other models slightly later, at 12-16 months (Moechars et al., 1999; Kulnane and Lamb, 2001). Double transgenics have mutations in APP and either presenilin-1 (PS1) or tau. These mice begin to develop Aβ between 2.5 months and 7 months (Borchelt et al., 1997; Holcomb et al., 1998; Lewis et al., 2001; Dinely et al., 2002; Schmitz et al., 2004). Triple transgenic mice have mutations in APP, PS1 and tau. They develop intraxonal Aβ at 3 months and extracellular Aβ at 6 months (Oddo et al., 2003). These transgenic animal models develop a substantial amount of Aβ deposits, which usually begin in the hippocampus and cortex, increasing in density and becoming more ubiquitous in several brain structures with age. While the exact distribution of plaques differs slightly between models, deposits usually occupy the frontal, temporal, and entorhinal cortex, in addition to the hippocampus, subiculum, and cerebellum (Games et al., 1995; Hsiao et al., 1996).

After the proven success of the mouse transgenic models, researchers sought to develop transgenic rats due to the larger size and ability to compare behavioral data to the already-established large database. In this study, we used both male and female triple-transgenic AD rats (Tg478/Tg1116/Tg11587) originally developed by Flood and colleagues (Flood et al., 2009), which were the first transgenic rats to produce extracellular Aβ plaques. This homozygous rat overexpresses 3 human genes: 1) human APP 695 with the K670N/M671L mutation; 2) human APP minigene with the K670N/M671L and V717F mutations; 3) human PS1 with theM146V mutation. Neuropathologically, sparse Aβ plaques begin to appear in the subiculum and CA1 regions of the hippocampus, as well as the cortex, between 7 and 9 months of age. Deposition significantly increases by 13 months (Figure 5) and seems to plateau by 18 months of age. Clearly, the plaque burden significantly increases with age, which has been validated with immunohistochemistry and *in vivo* PET imaging. The latter method uses an [F-18] FDDNP tag, which preferentially labels Aβ (Teng et al., 2011). Behaviorally, these rats perform approximately 50% worse than wild-type (WT) in the continuous spatial alternation task of spatial working memory between 3 and 10 months of age. They also spend less time exploring new objects in the novel object recognition task (NOR) between 8 and 17 months of age. Lastly, Morris water maze performance is significantly worse than WT in 3xTgAD rats 18-19 months of age (Teng et al., 2012 poster). It is important to note that this animal model does not produce neurofibrillary tau tangles; therefore we are restricted to only analyzing amyloidogenesis. However, since

studies suggest that Aβ overproduction precedes tau, focusing on the former is a justified first step.

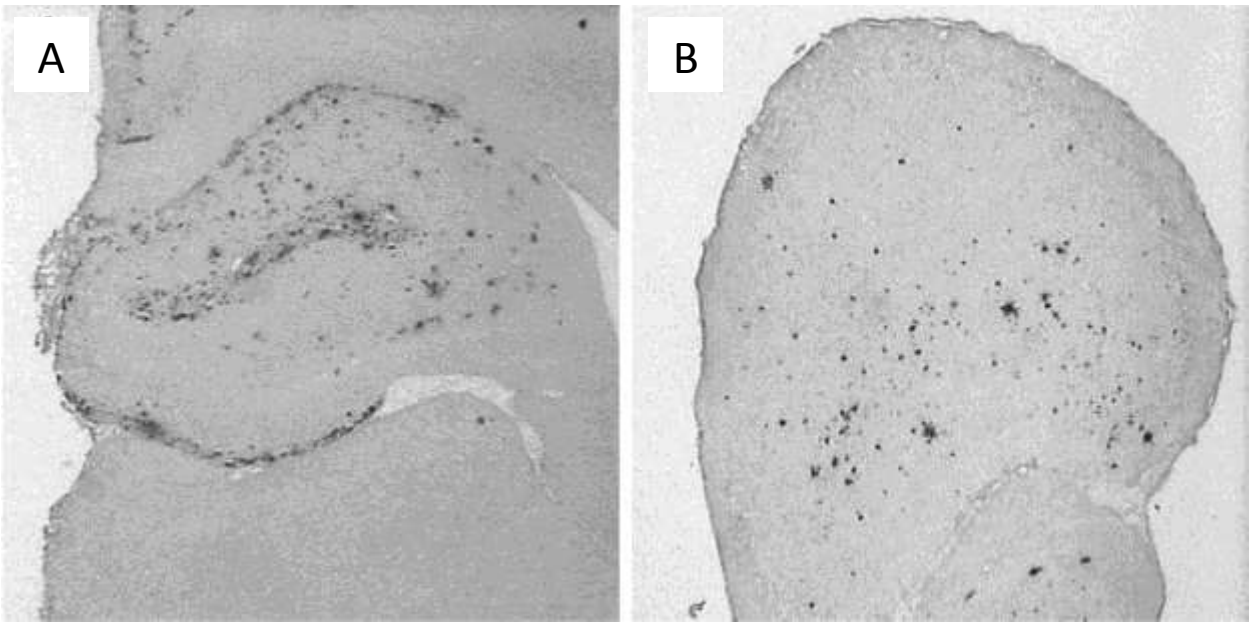

Figure 5. Aβ plaques with DAE staining in the A) hippocampus and B) frontal cortex of a 13-month old 3xTgAD rat (adapted from Teng et al., 2011)

SCOPE OF DISSERTATION

Repeat concussion in adolescents is a public health concern with so many young athletes participating in sport each year. In the 2013-2014 academic year, nearly 7.8 million student-athletes participated in high school sports in the United States, a number that has steadily increased since 1971 (National federation of state high school associations, 2014). Paralleling this trend, among collegiate sports, the rate of concussions increased 7% over a sixteen-year period, from the 1988-1989 season to the

2003-2004 season (Hootman et al., 2007). This trend may be due to an increase in the number of TBIs, increased youth participation in sport, or an increase in awareness. Regardless of the reason for this increase, participation and concussion rates among high school and college athletes are high enough to warrant attention. Furthermore, due to the brain's development during these adolescent years, it is critical that we understand the acute and chronic effects of repeat concussions.

The main theory explored in this thesis is based on a model proposed by Chen and colleagues (2004). Specifically, RTBI may cause traumatic axonal injury, which causes axonal transport to be impaired, leading to an accumulation of APP, BACE, and PS1 (Arfanakis et al., 2002; Smith et al., 1999). This increased substrate pushes the process toward the amyloidogenic pathway, where Aβ forms within the axon before being released and potentially aggregating in different parts of the brain. Therefore, RTBI in the adolescent rat brain may exacerbate amyloidogenesis later in development.

The vast majority of studies addressing chronic effects of RTBI utilize adult animal models. Since sport participation is highest in adolescence and professional athletes were initially at risk for TBI prior to their professional careers, it is important that we shift the focus to RTBI in the developing, adolescent brain. This is the first study to examine the long-term pathological consequences of RTBI in adolescence.

CHAPTER 1.

TO DETERMINE THE LONG-TERM EFFECT OF RTBI ON AMYLOIDOGENESIS

INTRODUCTION

Recent media attention has focused on the long-term cognitive effects of repeat mild traumatic brain injury (RTBI), causing alarm among the general population. Athletes and parents, in particular, are concerned about the chronic damaging effects of RTBI, but a causative link between RTBI and neurodegenerative disease has not yet been determined. In the past few years, several papers have been published on the effect of TBI on Alzheimer's disease (AD; Mortimer et al., 1985; Plassman et al., 2000), Amyotrophic Lateral Sclerosis (ALS; Chen et al., 2007; Schmidt et al., 2010), Parkinson's disease (PD; Goldman et al., 2006; Ben-Schlomo et al., 1997), and Chronic Traumatic Encephalopathy (CTE; McKee et al., 2009; DeKosky et al., 2013). Much of this concern comes from punch drunk syndrome, or dementia pugilistica (DP), a neurodegenerative disease first described in boxers in the 1920's (Martland, 1928). In the subsequent decades, DP was referred to as CTE, which was characterized clinically by motor problems with or without cognitive impairments. Pathological characteristics included cavum septum pallucidum, cerebellar cell loss, neurofibrillary tangles (NFTs) and substantia nigra degeneration (Roberts, 1969). Interestingly, Corsellis and colleagues

(1973) expanded the Roberts study dataset and detected amyloid beta in a fraction of the brains, concluding that CTE and AD share common pathologies and clinical symptoms. Modern cases of CTE describe a pathologically and clinically different disease with neuropsychiatric and behavioral symptoms such as depression and aggression appearing early, followed by memory deficits later in the disease process.

The majority of clinical RTBI papers have focused on CTE, but for several reasons it is important that researchers further explore the RTBI-AD relationship. First, recent reviews have highlighted the lack of epidemiological, cross-sectional, or prospective studies on RTBI among athletes and CTE (McCrory et al., 2013; Randolph, 2014; Maroon et al., 2015). Currently, the presence of CTE is only based on case studies. Second, there are no clearly defined pathological criteria for CTE. The main finding of NFTs does not distinguish CTE from other tauopathies. In fact, NFTs were identified in 97% of a large cohort of older adults without cognitive impairment. Furthermore, even among supposed CTE brains, the distribution of NFTs varies. Third, AD has a much higher incidence rate than CTE. As of August 1, 2013, there have only been 153 reported cases of CTE (Maroon et al., 2015) whereas AD affects over 4.7 million people in the U.S. (Hebert et al., 2013). Lastly, moderate to severe TBI is already a known risk factor for AD (Mortimer et al., 1985; Guo et al., 2000; Plassman et al., 2000). Contrary to CTE, AD is a highly prevalent and verifiable disease with a clear pathophysiological construct and an established association with TBI, rendering it an important area of focus for the long-term effects of RTBI.

Moderate to severe TBI is a known risk factor for AD, but a clear relationship between RTBI and AD has not yet been established. A meta-analysis of 15 TBI and Alzheimer's case-control studies revealed an odds ratio of 1.58, indicating a significant association between a history of TBI and the development of Alzheimer's disease (Fleminger, 2003). The inclusion criteria require loss of consciousness, however, which is not necessarily present in mTBI. AD, the most common form of dementia, is a neurodegenerative disease marked by memory loss and other cognitive impairments. The epidemiological connection between RTBI and AD is less clear, although one study found that retired NFL players with a history of repeat concussions had higher incidence rates of Alzheimer's disease under the age of 69 as compared to the average U.S. male population (Guskiewicz et al., 2003).

AD pathology has also been linked to TBI. AD brains contain two distinct pathological characteristics: extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. Aβ has been the most extensively studied hallmark characteristic of AD, probably due to the fact that the amyloid cascade hypothesis suggests that Aβ is the neurotoxic driving force of the disease. Aβ has been shown to cause the formation of neurofibrillary tangles as well as neuronal death, vascular damage, and dementia (Hardy and Higgins, 1992). There is a pathological link between moderate to severe TBI and AD, with Aβ found in brains of people who had a severe TBI (Ikonomovic et al., 2004). This pathological relationship has been examined in animal studies as well. Iwata and colleagues (2002) found that Aβ

accumulates in axonal bulbs up to one year post-fluid percussion injury. Sixteen weeks after a milder, but repetitive closed-skull injury in adult mice, Aβ was increased significantly in several regions, including the cortex and hippocampus. Aβ accumulates after TBI of all severities in the adult brain, but its time profile after repetitive mild injuries in the adolescent brain is unknown.

Human and animal mTBI research has largely focused on adults, but the higher rates of concussion in adolescence suggest a necessary redirecting. 70.5% of mild TBI occurs in adolescents between the ages of 10 and 19 (CDC MMWR, 2011). Repeat concussions in adolescents is a critical issue with 7.8 million students-athletes participating in sport in the 2013-2014 academic year (National federation of state high school associations, 2014) and concussions representing 8.9% of all high school sports injuries (Gessel, 2007). The cumulative effects of repeat concussions on acute symptomology in adolescence are clear, with symptom resolution time from repeat concussions significantly longer than from a new, single concussion. One study showed that among high school athletes, 0.6% of symptoms persisted for more than one month after a single concussion, but this percentage grew 10-fold (to 6.5%) after recurrent concussions (Castile et al., 2012). Furthermore, repeat concussions results in impaired attention and concentration (Moser et al., 2005), slower recovery time (Guskiewicz et al., 2003; Eisenberg et al., 2013), and slower processing speed (Gronwall et al., 1975) compared to cognitive functioning after a single concussion.

Gender and brain impact interval (BII) are two factors that should be considered in adolescent RTBI studies. Males and females do not respond to mTBI identically, which may have an impact on long-term consequences. High school females not only have a higher rate of concussion than male counterparts (Rosenthal et al., 2014), but they also experience more symptoms and have prolonged recovery times (Broshek et al., 2005). Gender differences also exist in Alzheimer's disease, with two-thirds of AD patients being women (Hebert et al., 2013). In animal models of AD, females develop more Aβ plaques than males (Callahan et al., 2001). Interestingly, however, there is no association between moderate to severe TBI and AD in females (Mortimer et al., 1985; Salib et al., 1997; Fleminger et al., 2003). The other important factor, BII, refers to the amount of time between repeat concussions. Studies have shown a period of metabolic brain vulnerability after a single concussion. If a second concussion occurs before the brain has recovered from the first, N-acetylaspartate (NAA), a marker for mitochondrial dysfunction, and concussion symptoms are worse than after a single injury alone (Vagnozzi et al., 2008). This finding was replicated in adolescent animal studies. After a mild, closed-head injury in the adolescent brain, the cerebral metabolic rate of glucose (CMRglc) transiently increases and then enters a period of depression, before returning to baseline by 3 days. If the second injury occurs during this CMRglc depression (e.g. 24 hours post-mTBI), the subsequent depression is greater in magnitude and duration. If, however, the second injury occurs after CMRglc returns to baseline (e.g. 120 hours post-mTBI), the depression profile is similar to that after a single injury (Prins et al., 2013). Experimental evidence on BII can inform return-to-play guidelines, which limit when an

athlete can resume his or her sport. If these evidence-based guidelines are adhered to, then athletes are in a much better position to protect their brains.

The majority of professional athletes do not experience their first concussion in adulthood, but rather these exposures often begin in childhood and adolescence. This reality, combined with the high rates of adolescent concussion, indicate that this age range should be the focus of more studies. Understanding the long-term effect of repeat concussions at varied intervals on AD pathology, specifically Aβ, can provide even more scientific evidence for the length of time required for an athlete to remain out of practice and competition. The purpose of this aim was two-fold: 1) to determine the long-term effect of adolescent RTBI with varied brain impact intervals on amyloidogenesis in adulthood, and 2) to compare this effect between male and female rats. We hypothesized that there would be no difference in Aβ burden between sham, 4RTBI24 or 4RTBI72 at 12 months of age, due to the mild severity of the injuries and the delayed follow-up time point, and that female rats would have more Aβ than males based on gender differences in plaque deposition in transgenic animals.

METHODS

Experimental design and subjects

Male and female postnatal day 35 (P35) triple-transgenic AD rats (3xTgAD) received sham (Male n=5; Female n=4), four injuries spaced 24 hours apart ($4RTBI_{24}$;

Male n=8; Female n=5), or four injuries spaced 72 hours apart ($4RTBI_{72}$; Male n=6; Female n=8). These time intervals were derived from previous studies showing that the rat brain metabolically recovers by 72 hours after RTBI with this model (Prins, 2013). Rats were aged to 12 months of age, and then sacrificed for immunohistochemistry (Figure 6).

Triple-transgenic AD rats (Tg478/Tg1116/Tg11587), originally developed by Flood and colleagues (Flood, 2009), were used in this study since rodents do not naturally produce Aβ plaques. This homozygous rat (3xTgAD) overexpresses 3 human genes: 1) human APP 695 with the K670N/M671L mutation; 2) human APP minigene with the K670N/M671L and V717F mutations; 3) human PS1 with theM146V mutation. The neuropathological timeline of these animals has been previously described (Flood et al., 2009; Liu et al., 2008). Generally, sparse Aβ plaques begin to appear between 7 and 9 months of age, particularly in the cortex and hippocampus. The plaque burden significantly increases with age (Teng, 2011). Breeding pairs were originally obtained from Cephalon Inc (West Chester, PA) and bred at the UCLA vivarium. The UCLA Animal Research Committee approved this study.

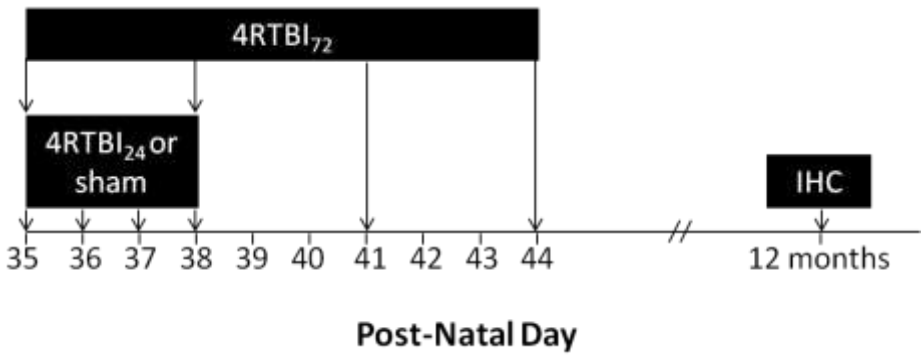

Figure 6. Experimental design aim 1. 3xTgAD P35 rats received sham, $4RTBI_{24}$, or $4RTBI_{72}$ and were sacrificed at 12 months of age.

Closed-head injury model

This injury model has previously been described in detail (Prins, 2010; Figure 7). Generally, under isoflurane (2.0%/100%O_2), the animal's head was shaven and placed against a wooden block within a stereotaxic frame without ear bars. An electronically-controlled pneumatic piston cylinder was mounted onto a stereotaxic micromanipulater to allow for precise localization of the impact center. A mask was used to mark the center of impact (-3AP, -4ML relative to bregma) and the injury tip was firmly zeroed against the skin. The piston was angled at 23 degrees away from vertical to allow the impactor to make contact perpendicular to the brain surface. The impactor tip (5 mm diameter) displaced the head 8mm at 36 PSI. The head was free to move in the direction of the injury. This level of impact does not cause skull fractures. However, it does produce apnea and delays the righting time (Table 4).

Figure 7. Closed-head injury model.

Tissue preparation

Animals were sacrificed 10.5 months post-RTBI (at 12 months of age) for immunohistochemical analysis. Rats were injected with a lethal dose of pentasol (100 mg/kg, IV) and perfused transcardially with a 10% sucrose solution, followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight and cryoprotected in 20% sucrose before frozen in methylbutane.

Immunohistochemistry

For DAE (rabbit polyclonal anti-Aβ1-13; Yang, 1994) immunohistochemistry, 40μm-thick sections were blocked with 0.5% H_2O_2 in phosphate buffer saline (PBS) for

30 minutes. Sections were then permeabilized with 2% normal goat serum (NGS; Vector Laboratories) plus 0.3% triton X-100 in PBS for 30 minutes and incubated in DAE antibody (1:600 dilution) at 4° overnight. After washing in PBS, sections were incubated for 1.5 hours in 1:500 biotinylated goat anti-rabbit secondary (Vector BA1000). Sections were incubated in ABC (Vector Laboratories) for 30 minutes and then DAB was applied. Sections were mounted and slides were dehydrated and coverslipped.

For GFAP, sections were blocked in 1.5% normal goat serum/0.1% bovine serum albumin, incubated for 1 hr in 1:2000 anti-GFAP (Abcam, ab5804), and blocked in 0.5% H_2O_2. For Iba1, sections were incubated in citrate for antigen retrieval, blocked in 0.5% H_2O_2, and incubated in 1:200 anti-Iba1 (Abcam, ab5076). For myelin basic protein (MBP) and neurofilament (NF), sections were incubated in 0.5% H_2O_2 to block endogenous peroxidase activity, permeabilized in 2% normal goat serum/0.3% triton X-100, and blocked in 1.5% normal goat serum. Then, they were incubated in 1:500 anti-MBP (Abcam, ab40390) or anti-NF (Millipore, MAB1621) overnight. After primary antibodies were applied, all sections were incubated in 1:200 biotinylated secondary antibody (Goat anti-rabbit, Vector BA1000; Goat anti-mouse, Invitrogen A21424; Horse anti-goat, Vector BA9500) for 30 minutes (GFAP, MBP, NF) or 2 hours (Iba1). After washing in PBS, sections were incubated in Vectastain ABC, developed with DAB, mounted, and allowed to dry overnight. Slides were then dehydrated and coverslipped.

Image J (National Institute of Health) was used to determine the amount of Aβ staining in the regions of interest (ROI). ROIs were manually drawn using anatomical

landmarks and the area stained was determined using thresholding analysis. Aβ burden was calculated by dividing the area stained by the total area of the ROI. Two brain sections from each animal were used in the analysis: 1) an anterior section, 0.2 anterior-posterior from bregma, and 2) an area under the injury site, -3.3 anterior-posterior from bregma.

Statistical analysis

All statistical analyses were performed using R software version 3.0.0 (R Development Core Team, http://www.R-project.org/; R Foundation for Statistical Computing, Vienna, Austria). An ANOVA with multiple factors was used to compare percent area (Aβ burden) in multiple regions-of-interest (ROI) and physiological responses between injury groups and between males and females.

RESULTS

Male and female rats were not statistically different in terms of weight, apnea, toe pinch, or righting after the last injury, so results were pooled. Righting time was the only physiological measure to differ significantly between injury groups ($p=0.03$). Righting time after $4RTBI_{24}$ was significantly longer (224 ± 17.1 s) than after sham (169 ± 15.4 s) or $4RTBI_{72}$ (208 ± 22.9 s), which were not different from each other (Table 4).

Injury group	Weight (g)	Apnea (sec)	Toe pinch (sec)	Righting (sec)
Sham	160 ± 11.9	x	x	169 ± 15.4
$4RTBI_{24}$	178 ± 10.5	2.4 ± 0.4	41 ± 1.8	**224 ± 17.1 ***
$4RTBI_{72}$	175 ± 9.8	1.6 ± 0.3	36 ± 1.8	208 ± 22.9

Table 4. Physiological response after the last injury. *p<0.05 compared to sham

For each region of interest (ROI), namely the hippocampus, entorhinal cortex, anterior parietal cortex, and posterior parietal cortex, the effect of RTBI on Aβ burden was compared in males and females using an analysis of variance (ANOVA) with multiple factors (i.e. injury, interval, gender, side).

In the hippocampus, there were significant main effects for injury (F=8.634, df=1, p=0.005) and interval (F=60.632, df=1, p=0). There were no main effects for gender (p=0.566) or side (p=0.791); therefore, males and females were collapsed, as were the ipsilateral and contralateral sides (Table 5). Aβ burden was significantly greater after $4RTBI_{24}$ (mean=3.62%) than sham (p=0; mean=1.32%) or $4RTBI_{72}$ (p=0; mean=1.00%). Additionally, Aβ burden was not statistically different between sham and $4RTBI_{72}$ (p=0.074; Figure 8; Figure 9).

	Df	Sum Sq	F	P-value
Injury	1	13.12	8.634	0.00476**
Interval	1	92.13	60.632	1.56e-10***
Gender	1	0.51	0.333	0.56617
Side	1	0.11	0.071	0.79100
Injury:Gender	1	0.30	0.197	0.65908
Interval:Gender	1	0.37	0.243	0.62373
Injury:Side	1	0.01	0.005	0.94315
Interval:Side	1	0.02	0.012	0.91423
Gender:Side	1	0.12	0.081	0.77662
Injury:Gender:Side	1	0.64	0.420	0.51956
Interval:Gender:Side	1	0.49	0.324	0.57172
Residuals	57	88.61		

Table 5. ANOVA table for Aβ burden in the hippocampus. *** p = 0; **p < 0.01

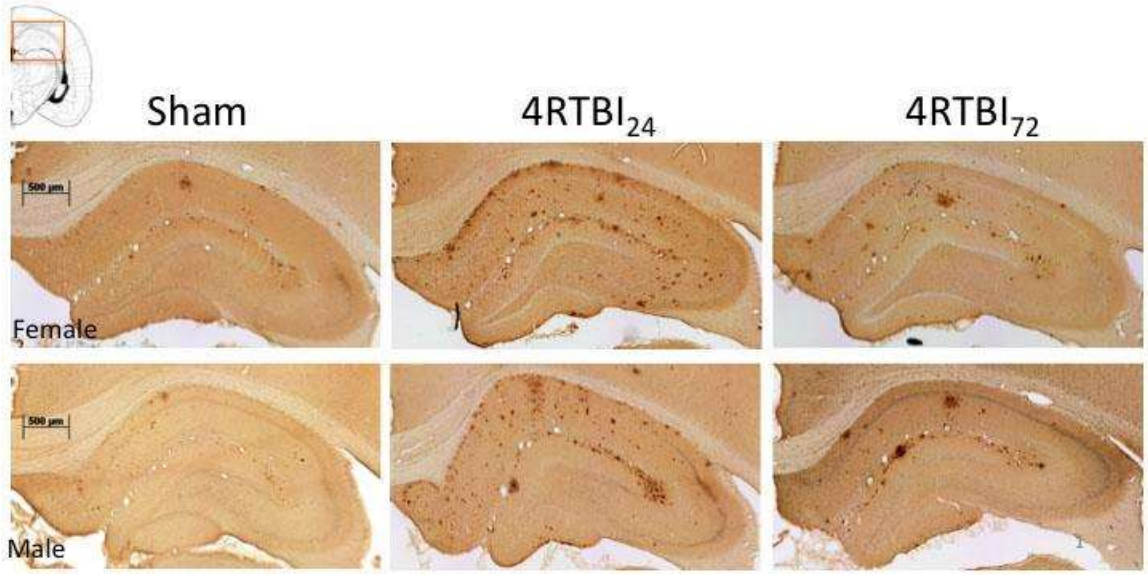

Figure 8. DAE staining in the ipsilateral hippocampus 10.5 months after sham, $4RTBI_{24}$ or $4RTBI_{72}$

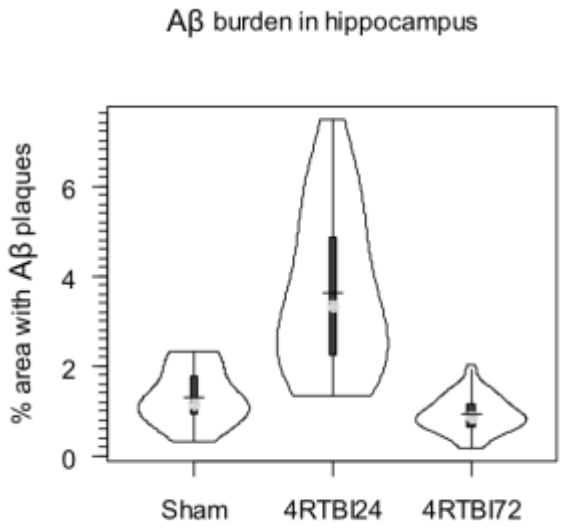

Figure 9. Violin plots of Aβ burden in the hippocampus. Horizontal line = mean. Grey dot = median. Vertical black bar = 25th to 75th quartiles.

In the posterior parietal cortex (Table 6; Figure 10B), there was a main effect for interval (p=0.0492), but a TukeyHSD post-hoc analysis did not reveal any differences in pairwise comparisons. This discrepancy may indicate a lack of power. There were no main effects for injury (p=0.4438), gender (p=0.3209), or side (p=0.5021).

	Df	Sum Sq	F	P-value
Injury	1	0.064	0.600	0.4438
Interval	1	0.440	4.160	0.0492*
Gender	1	0.107	1.015	0.3209
Side	1	0.049	0.460	0.5021
Injury:Gender	1	0.127	1.199	0.2813
Interval:Gender	1	0.013	0.127	0.7241
Injury:Side	1	0.041	0.387	0.5381
Interval:Side	1	0.008	0.074	0.7873
Gender:Side	1	0.002	0.018	0.8934
Injury:Gender:Side	1	0.001	0.006	0.9388
Interval:Gender:Side	1	0.000	0.000	0.9893
Residuals	34	3.597		

Table 6. ANOVA table for Aβ burden in the posterior parietal cortex. *$p < 0.05$

In the anterior parietal cortex (Table 7; Figure 10A), there were no main effects for injury (p=0.720), interval (p=0.261), gender (p=0.945), or side (p=0.179).

	Df	Sum Sq	F	P-value
Injury	1	0.23	0.130	0.720
Interval	1	2.28	1.298	0.261
Gender	1	0.01	0.005	0.945
Side	1	3.27	1.868	0.179
Injury:Gender	1	2.89	1.649	0.206
Interval:Gender	1	2.10	1.198	0.280
Injury:Side	1	0.29	0.163	0.688
Interval:Side	1	3.60	2.054	0.159
Gender:Side	1	0.00	0.003	0.959
Injury:Gender:Side	1	3.59	2.048	0.160
Interval:Gender:Side	1	1.01	0.577	0.452
Residuals	43	75.40		

Table 7. ANOVA table for Aβ burden in the anterior parietal cortex

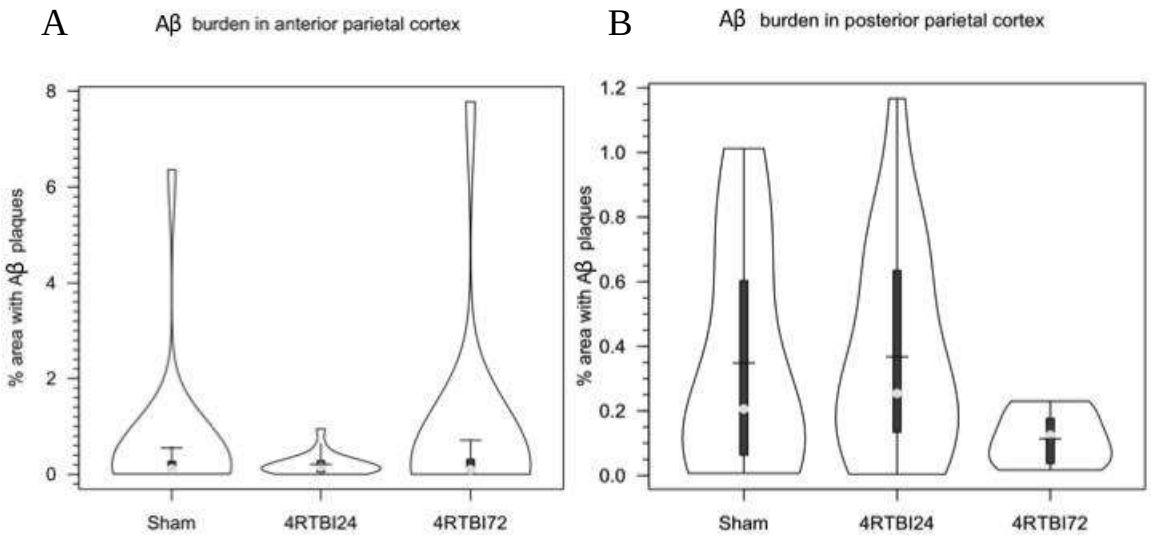

Figure 10. Violin plots of Aβ burden in the A) anterior parietal cortex and B) posterior parietal cortex. Horizontal line = mean. Grey dot = median. Vertical black bar = 25th to 75th quartiles.

In the entorhinal cortex (Table 8; Figure 11), there were no main effects for injury (p=0.0873), interval (p=0.0679), gender (p=0.6890), or side (p=0.3462).

	Df	Sum Sq	F	P-value
Injury	1	0.434	3.085	0.0873
Interval	1	0.498	3.537	0.0679
Gender	1	0.023	0.163	0.6890
Side	1	0.128	0.910	0.3462
Injury:Gender	1	0.032	0.229	0.6348
Interval:Gender	1	0.005	0.034	0.8539
Injury:Side	1	0.049	0.346	0.5597
Interval:Side	1	0.093	0.664	0.4204
Gender:Side	1	0.072	0.509	0.4799
Injury:Gender:Side	1	0.000	0.000	0.9829
Interval:Gender:Side	1	0.003	0.018	0.8933
Residuals	37	5.207		

Table 8. ANOVA table for Aβ burden in the entorhinal cortex

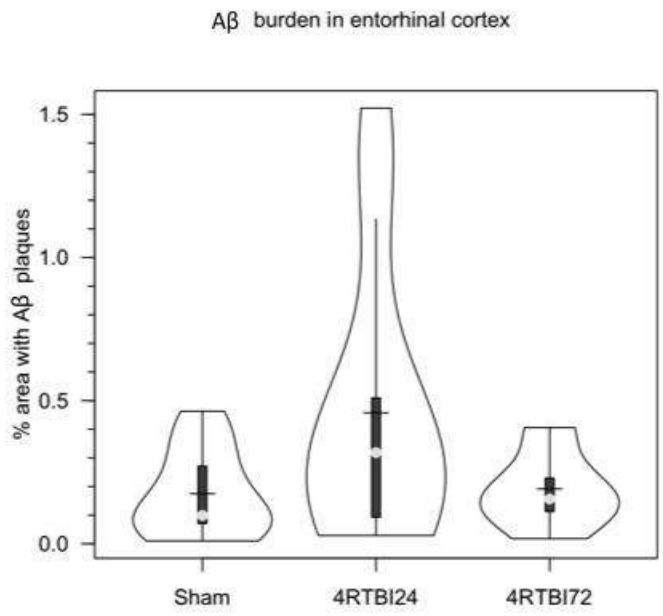

Figure 11. Violin plots of Aβ burden in the entorhinal cortex. Horizontal line = mean. Grey dot = median. Vertical black bar = 25th to 75th quartiles.

GFAP staining seems to be more robust 1 day after 4RTBI as compared to sham, especially in the molecular layer. These differences persist, but are not as pronounced, at the 1 week time point. By 8 months, astrocyte reactivity is similar in both the sham and 4RTBI animals (Figure 12).

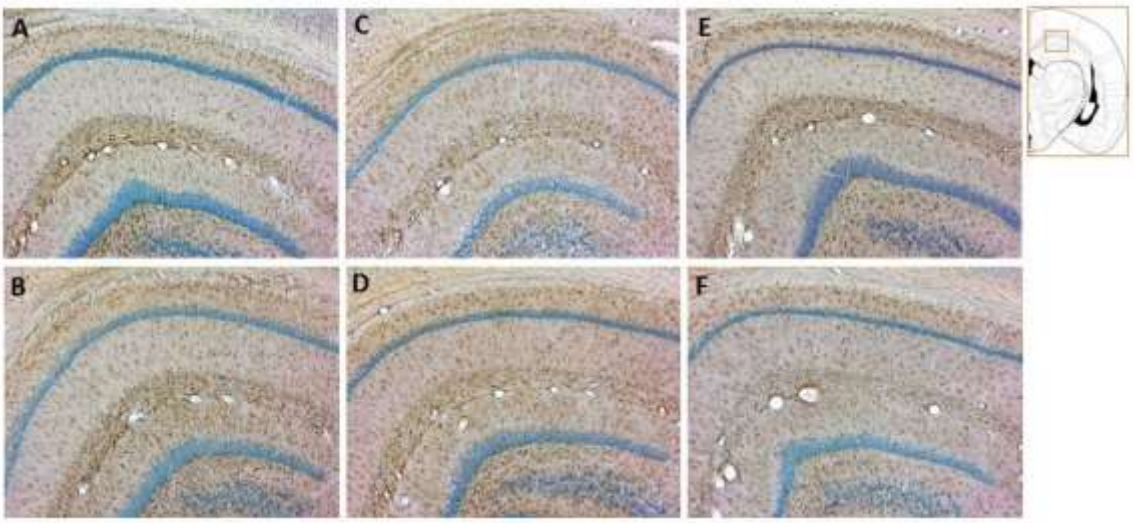

Figure 12. GFAP in the ipsilateral hippocampus after sham (A,C,E) and RTBI (B,D,F). GFAP burden is greater in the molecular layer 1 day after 4RTBI (B) than after sham (A). These differences persist at 1 week for 4RTBI (D) and sham (C), but are not present at 8 months (E,F).

Microglia activation, as assessed qualitatively by Iba1 staining, is more robust in the ipsilateral grey-white matter junction (Figure 13) and thalamus (Figure 14) 24 hours after 4RTBI versus sham. Sham and injured staining does not differ at the 1 week and 8 month time points.

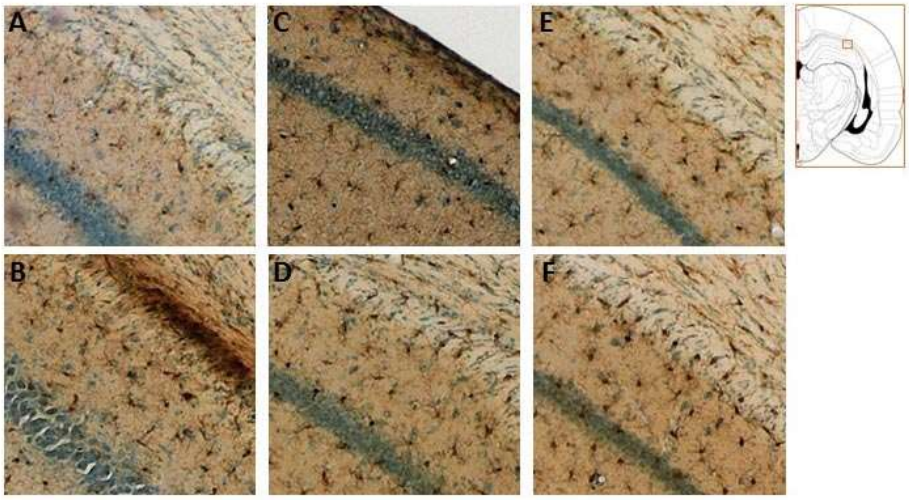

Figure 13. Iba1 staining in the ipsilateral grey-white matter junction after sham (A,C,E) and RTBI (B,D,F). Microglial activation appears to be more robust in RTBI at the 24 hour time point (B) compared to sham (A), but not at 1 week or 8 months.

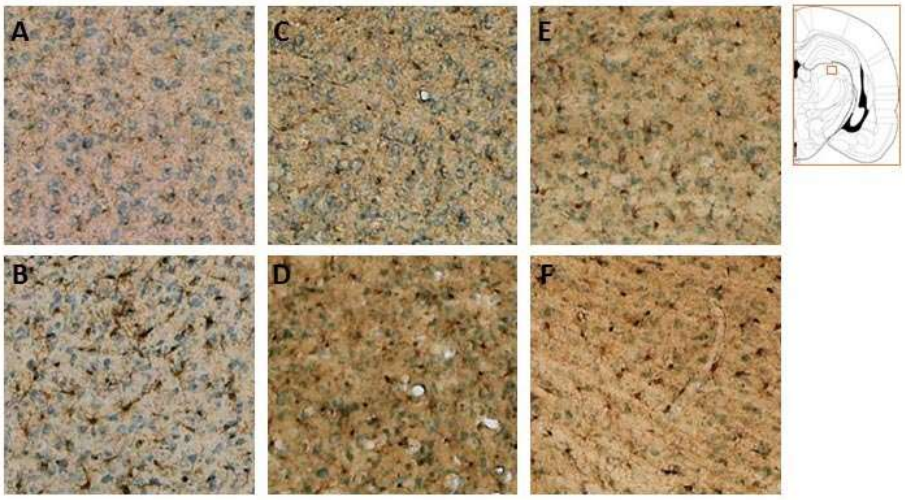

Figure 14. Iba1 staining in the ipsilateral thalamus after sham (A,C,E) and RTBI (B,D,F). Microglial activation appears to be more robust in RTBI at the 24 hour time point (B) compared to sham (A), but not at 1 week or 8 months.

DISCUSSION

The RTBI/AD relationship

Concerns over the damaging effects of repetitive mild TBI (RTBI) are at an historic high and the prevalence of AD is increasing, but previous studies evaluating the epidemiological link between mTBI and AD have been inconclusive (Plassman, 2000). This animal study is the first to address two central aims: 1) to measure Aβ pathogenesis several months after mild RTBI in the adolescent brain, and 2) to determine the effect of brain impact interval (BII) and gender on chronic Aβ accumulation. It also examines the potential mechanistic links between the two disease states.

In this study, we used a closed-head animal model and triple transgenic Alzheimer's rat to assess the effect of RTBI in the adolescent brain on long-term Aβ pathology. There was significantly more Aβ burden in the hippocampus compared to sham 10.5 months after repetitive injury spaced 24 hours apart. This finding is consistent with previous studies that show an increase in Aβ following TBI of various severities. However, these studies only examined changes in the adult brain (Roberts et al., 1994; Iwata et al., 2001; Ikonomovic et al., 2004; Johnson et al., 2012).

The increase in Aβ burden was region-specific and suggests acceleration in Aβ deposition. While plaques were apparent in the four regions-of-interest (ROIs) measured (entorhinal cortex, anterior parietal cortex, posterior parietal cortex, hippocampus), the

significant increase in Aβ burden, as compared to sham, was only seen in the hippocampus. RTBI does not seem to sprout Aβ plaques in new areas of the hippocampus. Rather, it exacerbates the normal pathological distribution of the plaques. In this 3xTg-AD animal model, plaques begin to appear in the subiculum and CA1 between 7 and 9 months of age (Flood et al., 2009). By 12 months, shams from the current study show deposits primarily in CA1. The $4RTBI4_{24}$ group, however, has Aβ in CA1, but also in CA2, which is very similar to the distribution in 13-month old rats of this model (Teng et al., 2011). These similarities between $4RTBI_{24}$ at 12-months and sham at 13-months suggests that RTBI exacerbates, and potentially accelerates, the normal distribution of Aβ plaques.

These findings were largely consistent with another mild RTBI study in adult transgenic mice, with some regional variations. Uryu and colleagues (2002) showed that Aβ burden increased 16 weeks after 2 repeat injuries spaced 24 hours apart. There were no differences at 2 days or 9 weeks post-injury, suggesting a delayed effect of RTBI on accelerated Aβ accumulation. Aβ staining occupied 1.5% and 3.6% of the total hippocampus area in the adult study and the current adolescent study, respectively. This % area translated to an approximately 8-fold increase in Aβ after RTBI versus sham in the adult study as compared to a 3-fold increase in the current study. While the animals in both studies were sacrificed at approximately the same age (12 and 13 months), there was a 6-month difference in the time post-injury. These differences suggest that there may be an age effect, with injuries in adolescents producing less Aβ chronically than

injuries in adulthood. While both studies showed increases in Aβ deposition in the hippocampus, Uryu and colleagues (2002) also detected accelerated pathology in the parahippocampal (entorhinal) cortex and the parietal cortex. In the current study, these regions showed some Aβ pathology, but it was not significantly greater than sham. These slight differences in regional Aβ deposition are not likely due to variations in injury severity, since both models produce axonal injury without overt cell death, as is evidenced by histological staining (Laurer et al., 2001; Prins et al., 2010). One possibility is the transgenic models used, since plaques often appear in slightly different patterns in different transgenic lines (Callahan et al., 2001; Liu et al., 2008). The most likely explanation, however, is the age at which the injuries were delivered. In the mouse study, injuries occurred at 9 months of age, the same time as when plaques also begin to appear (Hsiao et al., 1996). In our study, injury occurred several months prior to the beginning of plaque formation. There is a dearth of data relating the age at the time of injury to the risk of developing AD. It is possible that injuries in adulthood exacerbate the accumulation of plaques, but research needs to be conducted to address this question. Overall, these findings support the positive association between RTBI and accelerated amyloidogenesis at a chronic time point after injury.

Aβ burden was not significantly different between ipsilateral and contralateral hemispheres, which may be due to the biomechanics of the injury or the time delay between injury and Aβ analysis. The lack of hemispheric differences is consistent with findings from Uryu and colleagues (2002), who also used a repeat closed-head injury

model, albeit in adult mice. These results may reflect the diffuse nature of mild, closed-head injuries. Biomechanically, a human concussion is the result of rapid linear and rotational acceleration forces that can have a diffuse, rather than a focal, effect on the brain (Broglio et al., 2009; Rowson et al., 2012). This model produces a lateral injury, but depending on the outcome measure of interest, it can affect either one or both hemispheres. For example, in this injury model, axonal damage as measured by APP or DTI manifests laterally (Prins et al., 2010) whereas acute CMRglc depression appears bilaterally (Prins et al., 2013). Another possible, and even more likely, explanation is the time post-injury. AD is normally evolving in these 3xTg-AD rats, so even without TBI, Aβ pathology would develop bilaterally. Perhaps there might be more lateral plaque distribution if animals were sacrificed earlier, but they do not even begin to show some plaque deposits until 7-9 months of age. Preliminary analysis of 8-month tissue showed that those early plaques are sparse and bilateral differences are not seen. Considering the biomechanics of this injury, the time post-injury, and the normal, bilateral accumulation of Aβ, it is not surprising that hemispheric differences were not seen.

Contrary to our hypothesis, gender did not have an effect on amyloidogenesis after RTBI. Since most studies do not compare males and females, the hypothesis was based on the fact that female transgenic rats develop accelerated Aβ plaques than male rats, although this was only seen in older, 15-19 month old animals (Callahan et al., 2001). Moreover, AD disproportionately affects women as compared to men (Hebert et al., 2013). Two possibilities may explain the lack of a gender effect in this study: timing

and hormones. First, the rats in this study may have been sacrificed at an age during which pathological gender differences do not exist. The shams provide evidence for this explanation, with female and male shams exhibiting similar Aβ burden at 12 months of age. Unpublished data from our lab indicates that there may be age differences in the younger brain (9 months of age), with females developing plaques earlier than males. However, these trajectories seem to converge by 12 months of age, as was evidence by the lack of gender differences in shams. The second possibility is based on the effect of hormones. Estrogen has a protective role in cognitive decline (Siegfried et al., 2007), particularly due to its positive effect on the hippocampus (Ha et al., 2007; Ottowitz et al., 2008). This puts post-menopausal women at increased risk for cognitive decline (Jamshed et al., 2014). However, since rats do not go through menopause, this neurotrophic effect may persist with age in 3xTg-AD rats. Overall, this 3xTg-AD rat model seems to show gender differences much younger than in other models, and these differences are resolved by 12-months of age, potentially explaining the lack of a gender effect in amyloidogenesis.

The results of this study bolster previous findings that the brain impact interval (BII), or spacing between injuries, is critical in terms of chronic consequences of repetitive injury. Particularly noteworthy is the finding that BII has a significant effect on Aβ accumulation. Injuries spaced 24 hours apart increased Aβ, but when RTBI was spaced 72 hours apart, this effect was not seen. In fact, $4RTBI_{72}$ was not statistically different from sham. While the effect of BII on Aβ accumulation has not been previously

addressed, other studies have examined the importance of BII on other outcome measures, such as behavior. In a closed-head modified weight-drop model, mice that received 5 injuries with a BII of 1 day performed worse on the Morris water maze (MWM) compared to sham at the one month and one year time points after the last injury. When BII was increased to 1 month, MWM performance was not significantly different from sham. This study demonstrated that chronic behavioral deficits 1-year after RTBI could be ameliorated if the BII is extended from 24 hours to 1 month (Meehan et al., 2012). In the closed-head injury model used in this study, the metabolic recovery period after a single mTBI is 3 days (Prins et al., 2013). Here, we have demonstrated that chronic amyloidogenesis is not accelerated when repeat injuries occur 3-days apart, after the brain has metabolically recovered from previous injuries. BII seems to be critically important in terms of numerous chronic outcome measures, including AD pathogenesis and behavioral deficits.

Significance of plaque accumulation

The significance of Aβ plaques in AD, and therefore the TBI-AD relationship, remains unknown. While plaques are undoubtedly involved in the disease, their role in AD remains controversial. Two central possibilities exist: 1) Aβ plaques cause AD, or 2) Aβ plaques are the result of the Alzheimer's disease process. Recent literature suggests the verity of the latter, since AD begins to develop long before plaques appear (Kim et al., 2013). Furthermore, plaques have been found in 20-25% of older people

without dementia (Haroutunian et al., 1998; Price et al., 1999; Wolf et al., 1999; Bennett et al., 2006; Aizenstein et al., 2008). However, the level of insoluble Aβ does distinguish AD patients from controls (McLean et al., 1999). The somewhat ubiquitous nature of Aβ in the aging brain may provide support for why 99.6% of therapeutic approaches against AD, most of which were aimed at clearing Aβ plaques, have failed to halt cognitive decline (Cummings et al., 2014). Furthermore, recent evidence suggests that oligomeric Aβ, instead of plaques, better correlates with cognitive decline (Näslund et al., 2015) and synaptotoxicity (Walsh et al., 2002; Shankar et al., 2008). The role of soluble Aβ will be discussed in more detail in Chapter 3.

Although insoluble Aβ may not be as toxic as the soluble form, it still has negative influences. Plaques have correlated with microglial activation, suggesting that the presence of Aβ aggregates may perpetuate microglial reactivity, which is a known component of AD pathophysiology (Frautschy et al., 1998). Furthermore, these aggregations have been viewed as reservoirs, from which toxic soluble oligomers can be discharged (Haass and Selkoe, 2007).

Aβ plaques are not necessarily the end product of monomeric accumulation; rather, the various forms of Aβ represent a dynamic process whereby the peptide fluidly aggregates and disassembles (Upadhaya et al., 2012). The most accurate diagnosis of Alzheimer's disease, post-mortem, considers the total amount of Aβ, of which Aβ plaques are constituents (Upadhaya et al., 2012). Aβ plaques are undeniably characteristic of AD, and likely toxic, but they may be a secondary consequence of earlier

events, such as inflammation, astrocyte reactivity, or metabolic decline, all of which will be discussed in the next sections.

Mechanistic link between RTBI and AD

This study demonstrates a relationship between RTBI in adolescence and chronic amyloidogenesis, but a mechanistic explanation for this time gap has not yet been established. Specifically, are there changes in the post-RTBI brain that make it more vulnerable to Aβ aggregation? Several pathophysiological events are known to occur both after RTBI and before AD and these common pathways may help explain the positive correlation. Two of these processes, astrocyte reactivity and inflammation, were qualitatively assessed in this study. Since these experiments were the first to examine if RTBI in the adolescent brain can accelerate amyloidogenesis, the age at which Aβ burden would start to emerge was unknown. In a pilot study, we sacrificed the rats at 8 months of age (6.5 months post-RTBI) and failed to see differences between injury groups in Aβ burden. However, we stained this 8-month tissue, as well as 24-hour and 1-week tissue, for GFAP. Qualitatively, there seems to be a robust increase in GFAP reactivity 24 hours post-4RTBI$_{24}$, especially in the molecular and polymorphic region of the dentate gyrus. This difference is not detected at the 1-week or 8-month time points in this study, but increased astrocyte reactivity has been seen 1-week after 5 repeat, modified-weight drop injuries (Kane et al., 2012). Although not measured in this study, an increase in BII from 24 hours to 48 hours demonstrates comparable amounts of gliosis to that after a single

mTBI (Bolton, 2014). The increase in astrocyte reactivity is noteworthy because reactive astrocytes produce GABA in transgenic mice, which is known to impair memory, the hallmark behavioral correlate of AD (Jo et al., 2014). Although astrocyte reactivity is relatively transient, and may not explain long-term changes, perhaps it triggers another molecular sequence that is more persistent. In fact, studies have revealed a complex relationship between astrocytes and microglia, with astrocytes activating both pro- and anti-inflammatory cascades (Min et al., 2006; Farina et al., 2007; Sofroniew 2009).

Immunohistochemical analysis against Iba1, a marker for microglia, indicated increased inflammation in the injured hippocampus 24 hours and 1 week post-4RTBI$_{24}$. At the 8-month time point, inflammation was so ubiquitous in both sham and injured tissue that differences were unable to be detected. These results are similar to previous studies. In a closed-skull 2RTBI$_{24}$ study, injuries were delivered to 6-8 week old mice using a CCI device and microglia activation persisted up to 7 weeks after RTBI (Shitaka et al., 2011). At a more chronic time point of 30 days after a closed-head injury, microglia activation was not seen in the brains of mice who received 5 modified-weight drop injuries spaced 24-hours apart (Kane et al., 2012). Six and 12 months after closed-head RTBI in a non-transgenic adult mouse, closed-head RTBI did not result in increased inflammation at either 6 or 12-months post-injury (Mouzon et al., 2014). This chronic widespread inflammatory response in only the transgenic rat is likely due to the presence of Aβ plaques, which begin to appear at 7 months of age (Flood et al., 2009), because Aβ has been shown to induce inflammation (Jung et al., 2015). Furthermore, inflammation

can be self-propagating, with acute inflammation triggering more chronic inflammation (Lu et al., 2009). After a moderate TBI in young adult mice, inflammation increased acutely, but then returned to baseline by 7 days. Thirty days after injury, most microglia activation resolves, but a subset of primed microglia may persist and produce an exaggerated cytokine reaction in response to secondary immune challenges, such as subsequent injury, stressors, or even aging (Fenn et al., 2014). These studies suggest that astrocyte reactivity and inflammation occur acutely, with inflammation persisting in to the subacute phase after closed-head RTBI.

Depressed glucose metabolism, although not directly addressed in this study, is another overlapping molecular process between TBI and AD that may explain the link. As we have previously demonstrated with this animal injury model, the cerebral metabolic rate of glucose (CMRglc) increases transiently after injury, then enters a period of depression that is greater in magnitude and duration after repeat injuries spaced 24 hours apart than after a single injury (Prins et al., 2013). Metabolism depression recovered by 3 days after $2RTBI_{24}$, a pattern that is replicated when a repeat injury occurred after CMRglc recovered from the first injury. Although not yet studied, this recovery period may be longer after $4RTBI_{24}$. In humans, mTBI affects metabolic processes and decreases N-acetylaspartate (NAA), a marker of mitochondrial dysfunction. The NAA-to-Cr ratio was similar 30 days after a single concussion and in controls. However, there were differences between the double concussion cohort and controls 30 days post-injury (Vagnozzi et al., 2008). Although CMRglc depression does

not persist several months (in animals) or years (in humans) until the appearance of Aβ plaques, perhaps early metabolic impairment makes the brain more vulnerable to subsequent metabolic impairment later in life, in the period just preceding AD initiation. Could this be a self-perpetuating system where any perturbation in CMRglc reinforces and amplifies Aβ production? This question is starting to be addressed, with BACE acting as the mechanism by which metabolic depression increases Aβ accumulation (Struble et al., 2010).

The specific distribution pattern of Aβ plaques in the hippocampus may also provide insight in to the mechanism underlying the TBI-AD relationship. AD is a synaptic transmission disease with synaptic damage occurring early in the neurodegenerative process (Masliah, 2000; Crews and Masliah, 2010). An increase in Aβ deposits in the molecular layer, as was observed in this study, may be significant because past studies have shown, through electron microscopy, a significant spatial relationship between synaptophysin-positive boutons and Aβ plaques as well as a decrease in synaptic density in this region in aged transgenic mice (Dong et al., 2007). Furthermore, in this study there was robust labeling of Aβ deposits in CA1 and CA2, areas of the hippocampus that are particularly vulnerable to TBI (Baldwin et al., 1997; Norris and Scheff, 2009). Understanding the localization of potentially toxic Aβ plaques in the hippocampus may help explain how disruption in hippocampal circuitry after RTBI might affect memory, the most common behavioral correlate of AD.

Other possible mechanisms

The risk of developing AD after TBI is likely due to multiple factors, some of which were not explored in this study, including but not limited to dysfunctional clearance mechanisms and genetic susceptibility. Aβ production is a normal physiological process (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992), but in a subset of people, Aβ is either over-produced or inefficiently cleared. Microglia express receptors involved in Aβ clearance, but in older transgenic mice, these receptors are under-expressed compared to those in younger mice (Hickman et al., 2008). This indicates an age-dependent difference in Aβ clearance, which may be further impaired by brain injury. Another potential risk factor for accelerated AD development after TBI is APOE ε4. APOE ε4 is a genetic risk factor for sporadic AD and may work by affecting clearance of Aβ (Castellano et al., 2011). In an animal study, APOE ε4 was shown to influence the deposition of Aβ after TBI (Hartman, 2002). APOE ε4 mice also demonstrated increased $A\beta_{40}$ with age, a trend not seen in wild type mice (Mannix et al., 2011). However, the relationship between APOE ε4 and TBI is inconclusive with another study indicating no effect of the APOE ε4 allele after RTBI (Mannix et al., 2013). The APOE ε4 allele involvement in Aβ clearance and the RTBI-AD relationship is complex, but warrants increased attention.

Limitations

This data holds translational value for athletes who sustain repeat concussions, but it must be interpreted with caution. The rats used in this study are predisposed to develop one of the hallmarks of AD, amyloid beta plaques. While this study does not address whether or not RTBI can cause AD pathogenesis in the rodent, it does suggest that RTBI in the adolescent brain might exacerbate AD pathogenesis in a brain already at risk for developing the disease. In human studies, people with a history of TBI had an earlier onset of AD symptoms than those without head injuries (Gedye, 1989; Nemetz, 1999). The results of this study provide prospective, pre-clinical support for Guskiewicz and colleagues' (2003) epidemiological study, which found that NFL retirees with a history of repeat concussions expressed higher rates of late-life cognitive impairment and Alzheimer's disease than age-matched controls. Perhaps Aβ itself, or the molecular pathways it activates, contributes to behavioral impairments.

This study does have some limitations. First, as was mentioned earlier, these transgenic rats are genetically modified to consistently develop Aβ pathology and behavior. The rats used in this study overexpress three human genes, two APP genes and one in PS1 gene, which mimics the very rare early onset familial form of AD. Transgenics are the best animal models to study Alzheimer's pathogenesis since rodents do not naturally develop abundant Aβ plaques (Pierce et al., 1996). For these reasons, this study cannot address the causal effect of RTBI on AD. However, it does examine the relationship between RTBI and accelerated AD pathology. The second limitation is the

lack of tau quantification, the second pathological hallmark of AD. Unfortunately, phosphorylated tau was impossible to measure because it is not produced in this transgenic model. While the relationship between Aβ and tau is complicated, Aβ has been shown to accelerate tau deposition (Pooler et al., 2015). Since we have now shown a correlation between RTBI and increased Aβ burden, it is reasonable that tau would increase chronically after RTBI as well. In one study, TBI independently increased tau acutely after CCI injury, but chronic effects were not examined (Tran et al., 2011). The third limitation is the lack of behavioral tests, which might help explain the significance of increased Aβ burden. Standard behavioral tests are not feasible with these cognitively impaired transgenic rats at older ages. In a pilot study, 8-10 and 12-13 month old transgenic rats performed significantly worse than sham in the Novel Object Recognition task, a measure of recognition memory (Teng et al., 2012). Since the sham rats already perform at near chance levels during this advanced stage of disease progression, and performance cannot be worse than chance, an injury effect would be impossible to detect. Future studies should incorporate behavioral tests to determine whether or not the amount of plaques correlates with behavioral dysfunction, but would need to do so at slightly earlier ages. In transgenic mice, performance on the radial arm water maze was significantly worse for animals that received a moderate CCI compared to sham (Tajiri, 2013). Furthermore, since cognitive impairments begin prior to plaque formation (Smith, 1998), behavior should be analyzed at a pre-pathological stage, which might correspond to 7 months for these 3xTg-AD rats.

Conclusions

This study addresses the critical question of return-to-play by demonstrating the effect of BII on long-term AD pathogenesis. Most clinical studies have relied on retrospective self-reports of concussion history, which is inherently biased. Previous experimental studies have shown that increasing the interval between injuries decreases cognitive deficits (Longhi, 2005; Meehan, 2012; Prins, 2013). To our knowledge, this study is the first to demonstrate decreased chronic Aβ pathology with a greater time interval between injuries. While the cognitive effect of reduced Aβ is unknown, the finding that Aβ burden is greater than both sham and 4 injuries spaced 72 hours apart gives merit to return-to-play guidelines that keep an athlete out of physical activity at least until symptoms have resolved. This study represents histological validity for strict return-to-play guidelines. In summary, we demonstrated that repetitive mild TBI with a shortened BII during adolescence is correlated with accelerated, insoluble Aβ deposition later in life. However, if the BII increases and the RTBIs are spaced farther apart, this increase in Aβ is not seen. These findings reinforce the importance of return-to-play guidelines on the long-term neuropathological health of adolescent athletes. Although the mechanism underlying this RTBI-AD relationship is unknown, inflammation and metabolic depression are molecular pathways common to both RTBI and AD. These processes, in addition to white matter damage, which will be discussed in the next chapter, may provide us with an understanding of how RTBI and AD are linked.

CHAPTER 2.

TO DETERMINE ACUTE AND CHRONIC WHITE MATTER CHANGES AFTER RTBI

INTRODUCTION

Traumatic axonal injury (TAI) may be the mechanism by which RTBI exacerbates Alzheimer's disease (AD) pathogenesis. AD is a neurodegenerative disease, progressing over several decades, and ultimately leading to loss of brain tissue and significantly diminished cognitive functioning. AD and mTBI have common molecular and pathophysiological pathways and the acute events, such as TAI, after repetitive overlapping mTBI may induce some of these shared processes.

The high prevalence of mTBI in adolescents, combined with the exaggerated symptoms after RTBI and the concern over long-term consequences justifies exploration of the neuroanatomical changes occurring both acutely and chronically after RTBI. The incidence of RTBI in adolescents is difficult to ascertain with many concussions going unreported. However, it is estimated that between 5.6% and 36% of concussions in high school and college sports are repeat insults, although most of this data was acquired in football (Collins et al., 1999; Langburt et al., 2001; Moser et al., 2005). Approximately 80% of concussion symptoms resolve within 7-10 days (Meehan, 2010; Macciocchi,

1996), but 10-17% of individuals can have symptoms that persist for weeks or even months (Makdissi et al., 2010; McCrea et al., 2013). These symptoms may be due to, or at least initiated by, underlying TAI.

One way to measure TAI after RTBI is with diffusion tensor imaging (DTI). Imaging concussions is a challenge because the structural damage is too subtle to appear on standard scans. Conventional imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) return negative results after mTBI (Bazarian et al., 2007; Hughes et al., 2004; Arfanakis et al., 2002). However, diffusion tensor imaging (DTI) has been proposed as a powerful method that is sensitive enough to detect axonal injury (Brandstack et al., 2013; Mac Donald et al., 2007; Bazarian et al., 2007; Arfanakis et al., 2002) and to predict outcome (Yuh et al., 2014) after mTBI. DTI quantifies the direction of water diffusion to provide information about the microstructure of axonal bundles.

There are several ways to analyze DTI data, with new methods emerging frequently. The two main categories of analysis are region-of-interest (ROI) and whole-brain. ROI analysis techniques involve either manually drawing regions or aligning the brain to an atlas and extracting the ROIs defined in the atlas. Manual ROIs are problematic due to the low resolution of DTI and the inability to see small regions. Both manual- and atlas-based ROI methods are time consuming, rendering it impossible to analyze the entire brain (Snook et al., 2007; Jones and Cercignani, 2010). Whole-brain imaging techniques, alternatively, are automated and compute measurements on a voxel-

by-voxel basis by registering diffusion maps to a standard space. The two predominant types of whole brain analysis are voxel-based morphometry (VBM) and tract-based spatial statistics (TBSS). VBM assumes perfect registration and attempts to accomplish this by smoothing any areas of misalignment. The lack of true alignment and the process of smoothing can have profound effects on the data (Jones et al., 2005). TBSS improves upon VBM by eliminating standard registration algorithms and instead projecting FA maps on to a mean, skeletonized FA map before calculating voxelwise comparisons (Smith et al., 2006; Figure 14). TBSS has been successfully applied to TBI data (Yuh et al., 2014; Zhang et al., 2010).

Unlike other parameters that are measured with ROI or VBM analysis techniques (axial diffusivity, radial diffusivity, and mean diffusivity), TBSS focuses on fractional anisotropy (FA), the most commonly used DTI metric. In white matter, water movement is restricted along the length of the axons due to myelin sheaths. FA is a proxy for white matter microstructure and refers to the directional diffusion properties of the water molecules. It ranges from 0 to 1 with "0" representing unrestricted diffusion, equal in all directions and "1" representing complete anisotropy with diffusion in only one direction.

DTI studies after mTBI are limited, but reveal somewhat conflicting results. Acutely, the majority of clinical studies in adolescents show an increase in FA (Wilde et al., 2008; Chu et al., 2010; Bazarian et al., 2012), but two other studies demonstrated no changes in FA (Cubon et al., 2011; Maugans et al., 2012). This increased FA seems to persist up to two months post-mTBI (Virji-Babul et al., 2013). Chronic studies have only

been performed in animal models of RTBI, which focus entirely on adult animals. One animal study measured DTI 60 days after RTBI and found an increase in radial diffusivity, which is often coupled with a decrease in FA. However, it is important to note that the injury was of moderate severity, involving a craniotomy and a CCI device (Donovan et al., 2014). Six months after milder RTBI, adult mice showed no differences in DTI metrics, indicating a lack of white matter alterations (Mannix et al., 2013). A meta-analysis of DTI studies after mTBI, although not necessarily RTBI, found that elevated FA values are most often shown acutely (< 2 weeks) after mTBI, whereas decreased anisotropy is noted in the subacute or chronic phases (> 2 weeks; Eierud et al., 2014). Overall, there seem to be time- and mechanism-dependent changes in white matter microstructure after injury that may show a different profile after multiple injuries.

There are several strong possibilities for the lack of consensus in the literature. First, the number of previous concussions varies significantly, from studies failing to report injury history to others grouping those with a single mTBI together with those having had multiple concussions. Second, the mechanism of injury is not consistent within or between studies. Some subject pools included adolescents with "subconcussive hits" (Cubon et al., 2011) and others combined high-speed motor vehicle accidents with sport concussions. Third, the duration between injury and DTI acquisition ranges from 24 hours to less than 1 month for acute time points and 1 month to 6 months for chronic time points. These inconsistencies very likely contribute to the conflicting data and might be avoided with the implementation of RTBI studies in adolescent animals.

The interpretation of DTI results after TBI remains unclear, although some studies have attempted to correlate DTI indices with histological changes. Budde et al. (2011) showed that a transient increase in FA after CCI injury was correlated with reactive astrocytes (i.e. gliosis) and a subsequent decrease in FA was attributed to demyelination and axonal degeneration. In another CCI study, an acute decrease in FA and axial diffusivity (the amount of diffusion parallel to the axon) corresponded with axonal injury and a subacute increase in radial diffusivity (average diffusion perpendicular to the axon) corresponded with demyelination and edema (Mac Donald et al., 2007). Clearly, the neuropathological correlates of DTI metrics may change throughout the post-injury phase and the age effect has not yet been explored.

Little is known about the chronic effect of RTBI in the adolescent brain. In our efforts to understand some of the acute changes after RTBI that may contribute to accelerated, long-term Aβ accumulation, we used DTI to examine white matter microstructure at 1 week and 6 months after RTBI in adolescent rats. In contrast to the transgenic animals required for use in chapter 1, non-transgenic wild-type Sprague-Dawley rats were used for this study. At the time these experiments were conducted, the transgenic breeding colony was very small and would not support the sample sizes needed for each of the four injury groups: sham, single mTBI (TBI), double mTBI (2RTBI), and quadruple mTBI (4RTBI). We hypothesized that fractional anisotropy would decrease sub-acutely in a dose-dependent manner following mTBI, before returning to sham levels by 6 months post-injury. We also hypothesized that these

changes would correlate with markers of axonal degeneration and occur predominantly in the corpus callosum and external capsule, particularly in the region under the injury site.

METHODS

Experimental Design and Subjects

Postnatal day 35 (PND35) male Sprague-Dawley rats underwent sham, single, double (2RTBI), or quadruple (4RTBI) injuries. Repeat injuries (2RTBI and 4RTBI) were spaced 24 hours apart. Rats were scanned in vivo with diffusion tensor imaging (DTI) 1 week after TBI and sacrificed 24 hours after scanning for immunohistochemistry. Another set of rats were scanned with DTI 6 months after TBI and sacrificed 24 hours later (Figure 15). All procedures were approved by the UCLA Chancellor's Committee for Animal Research.

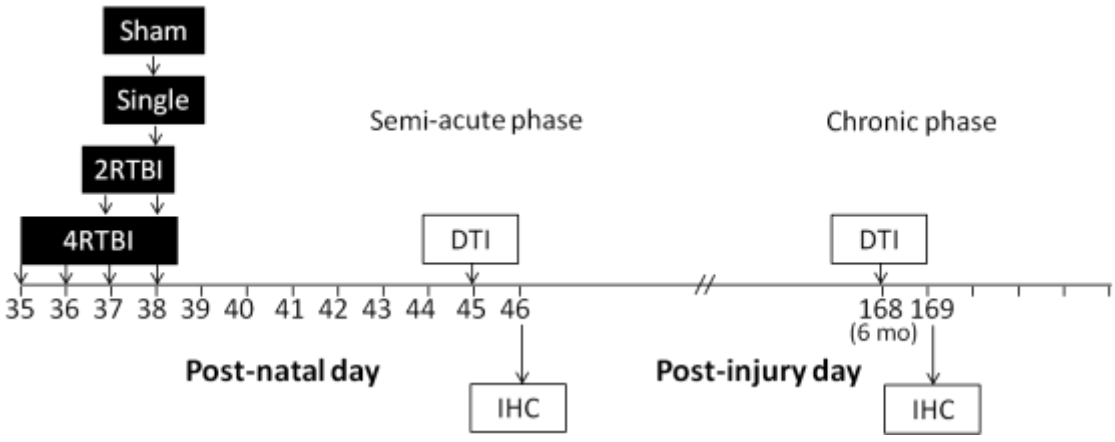

Figure 15. Experimental design aim 2

Closed-head injury model

Same as in chapter 1.

Diffusion tensor imaging

Acquisition: Rats were anesthetized with isoflurane (3% induction and 0.5–1% maintenance) and placed in an MR-compatible stereotaxic frame within a 7.0T scanner (Bruker Biospin 7.0 Tesla). Respiration was monitored with two fiber optic cables to detect the chest motion of the animal. The signal was fed into an oscilloscope that displayed the chest motion for monitoring throughout the experiment. Body temperature was maintained by circulating warm air around the animal. The imaging protocol consisted of a RARE anatomical scan and a DTI scan with the following shared geometric settings: Field-of-view (FOV) = 35x35mm; image dimension = 128x128; slice thickness = 0.75mm. The Epi spin-echo DTI sequence had the following parameters: number of slices = 25 and number of gradient encoding directions = 34. Four repetitions were used to improve signal-to-noise ratio. Total imaging time was 1 hour per animal.

Processing: Images were initially processed one of two ways, depending on the need for motion correction. For images without motion, tensor maps were immediately computed. For images with motion, the motion tensors were deleted and then FA maps computed using DTI studio (version 3.0.3).

Analysis: Whole-brain tract-based spatial statistics (TBSS; FSL version 4.1, Functional MRI of the Brain [FMRIB] Software Library [FSL]) was used to analyze the white matter (Figure 16). The detailed procedure has been described in Smith et al. (2006). Briefly, each subject's FA image was transformed to a single subject's (target) space using FLIRT software (FMRIB Software Library, Oxford, UK) an affine (linear) registration tool. Then, a mean FA skeleton was created, which represents the center of all white matter tract bundles common to all subjects. The mean FA skeleton was thresholded to include only voxels with FA > 0.28. All aligned FA data was then projected on to the skeleton, so that each skeleton voxel is filled with FA values from the nearest local tract center. Voxelwise statistics were computed across subjects in the skeleton space.

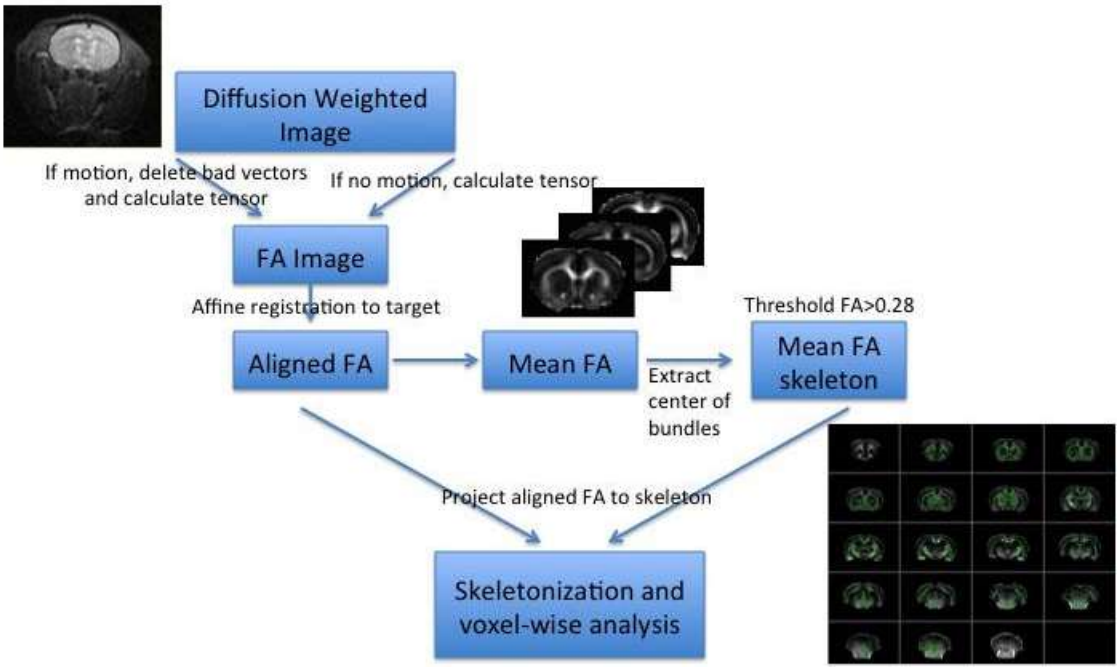

Figure 16. Whole-brain tract-based spatial statistics (TBSS). Flowchart shows image analysis protocol beginning with the raw, acquired diffusion weighted image.

Immunohistochemistry

Animals were euthanized 24 hours after either the 1-week or 6-month DTI scan and perfused with 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight, cryoprotected in 20% sucrose, and cut in 40μm coronal sections. For GFAP, sections were blocked in 1.5% normal goat serum/0.1% bovine serum albumin, incubated for 1 hr in 1:2000 anti-GFAP (Abcam, ab5804), and blocked in 0.5% H_2O_2. For Iba1, sections were incubated in citrate for antigen retrieval, blocked in 0.5% H_2O_2, and incubated in 1:200 anti-Iba1 (Abcam, ab5076). For myelin basic protein (MBP) and neurofilament (NF), sections were incubated in 0.5% H_2O_2 to block endogenous peroxidase activity, permeabilized in 2% normal goat serum/0.3% triton X-100, and blocked in 1.5% normal goat serum. Then, they were incubated in 1:500 anti-MBP (Abcam, ab40390) or anti-NF (Millipore, MAB1621) overnight. For APP, sections were dehydrated in95% ethanol/5% acetic acid, permeabilized in 2% normal goat serum/0.3% triton X-100, and blocked in 1.5% normal goat serum. They were then incubated in 1:200 anti-rabbit APP (Invitrogen 51-2700). After primary antibodies were applied, all sections were incubated in 1:200 biotinylated secondary antibody (Goat anti-rabbit, Vector BA1000; Goat anti-mouse; Horse anti-goat, Vector BA9500) for 30 minutes (GFAP, MBP, NF, APP) or 2 hours (Iba1). After washing in PBS, sections were incubated in Vectastain ABC, developed with DAB, mounted, and allowed to dry overnight. Slides were then dehydrated and cover slipped.

Statistics

DTI was analyzed with voxelwise tract-based spatial statistics. Immunohistochemistry analysis was performed using R software version 3.0.0 (R Development Core Team, http://www.R-project.org/; R Foundation for Statistical Computing, Vienna, Austria). Analysis of variance (ANOVA) with multiple factors was used to compare percent area stained in multiple regions of interest for group (sham, single, 2RTBI, 4RTBI). Tukey post-hoc analysis was performed when necessary. P-values less than 0.05 were deemed significant.

RESULTS

Experiment 1: DTI

An F-test of the TBSS-derived FA data (Figure 17) indicated that there was not a main effect for injury group at either 1 week ($p=0.3$) or 6 months ($p=0.4$). A post-hoc analysis with a planned comparison of 4RTBI < sham indicated significant differences in the anterior midline corpus callosum. Additionally, in the sections under the injury site, there were significantly different voxels in the ipsilateral corpus callosum, external capsule and fimbria (Figure 18).

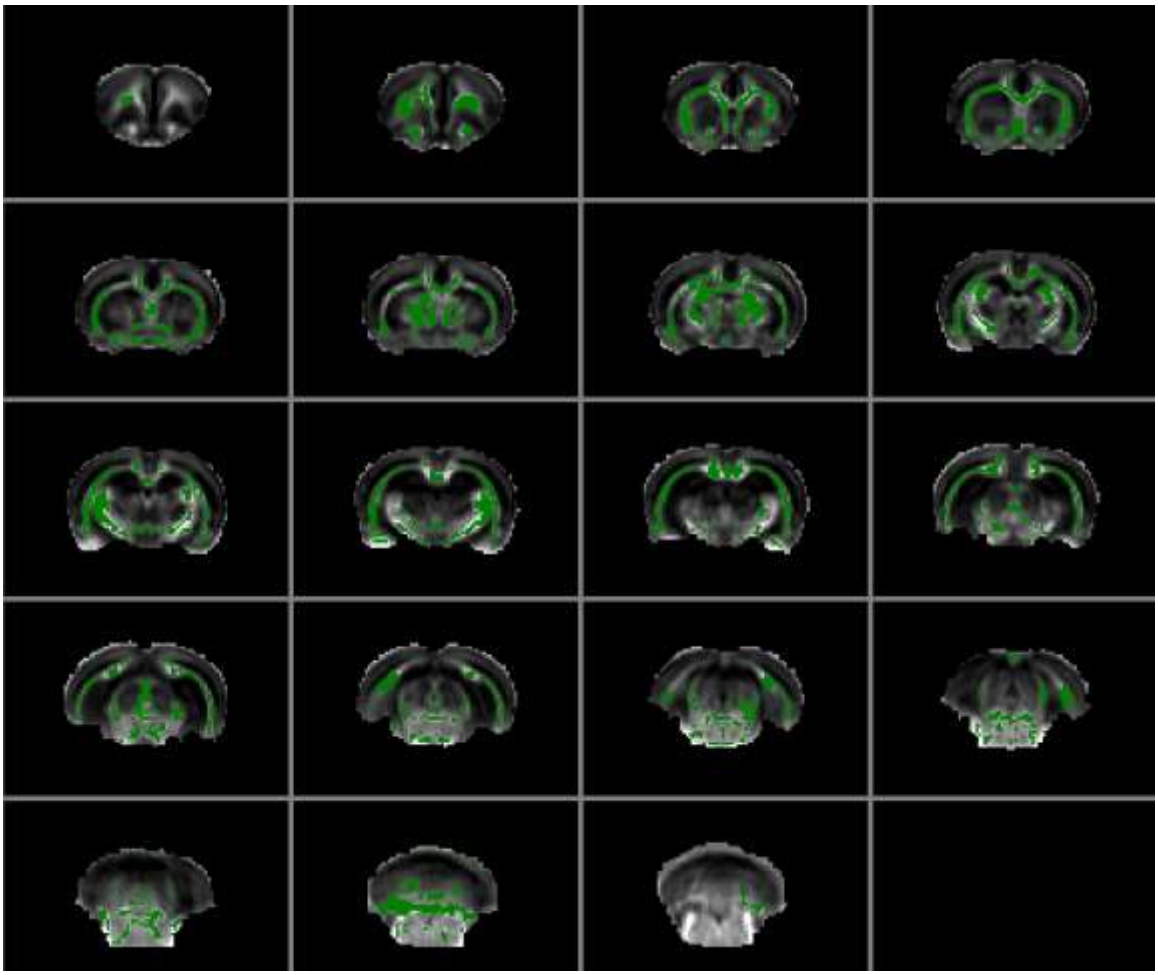

Figure 17. Results of an omnibus F-test comparing the mean FA within each voxel between sham, single, 2RTBI, and 4RTBI at 1 week. Green represents the mean FA skeleton. There is no red, orange, or yellow overlay, indicating no significant differences. Coronal sections are anterior to posterior from the top left to the bottom right. The left side of each image corresponds to the ipsilateral hemisphere.

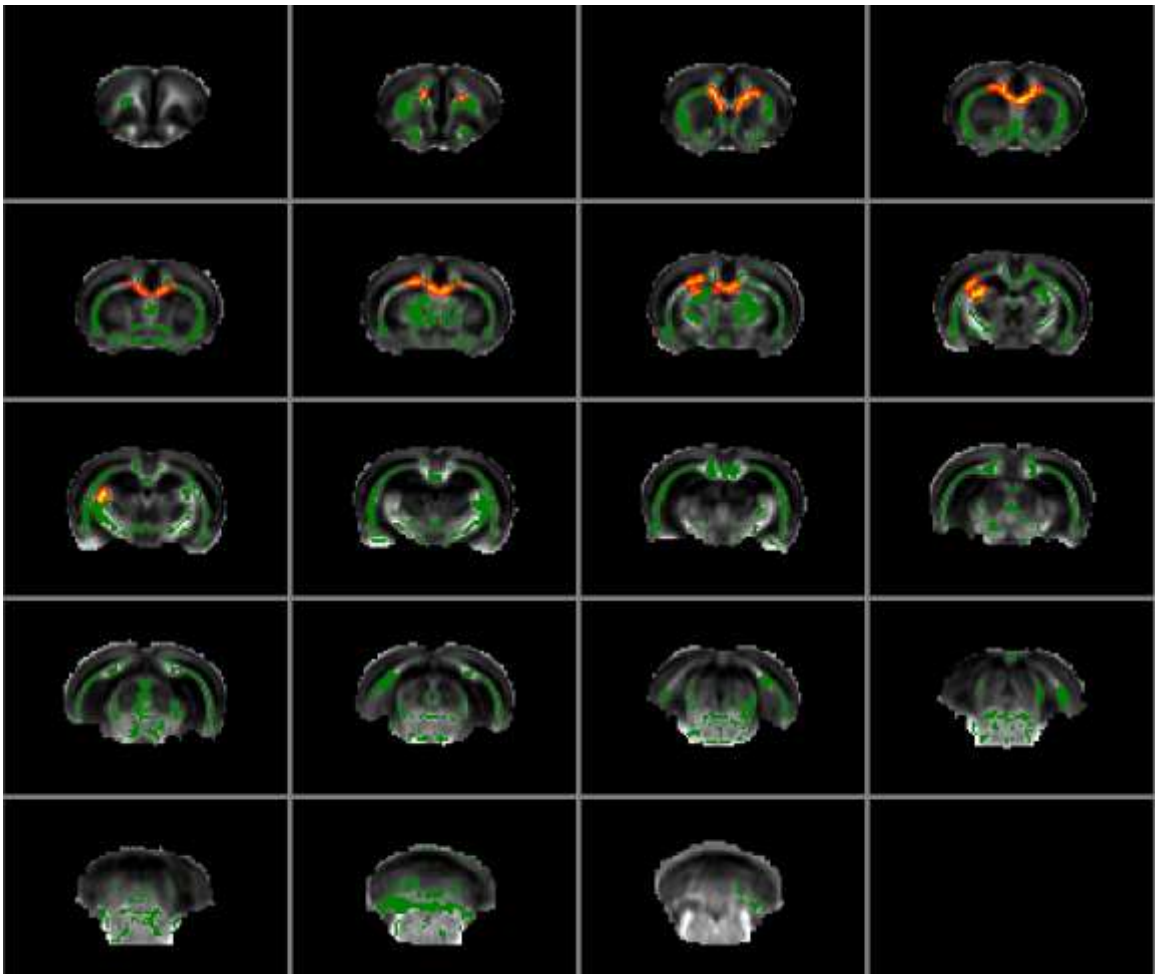

Figure 18. Results of paired comparisons from the TBSS analysis of the mean FA within each voxel between sham and 4RTBI at 1 week post-TBI. Green represents the mean FA skeleton. The red and yellow indicate significantly decreased FA in the corpus callosum, external capsule and fimbria after 4RTBI compared to sham. Coronal sections are anterior to posterior from the top left to the bottom right. The left side of each image corresponds to the ipsilateral hemisphere.

Experiment 2: Immunohistochemistry

Immunohistochemistry was used to visualize GFAP, Iba1, APP, and MBP and NF in the brain tissue collected at 1 week and 6 months post-TBI. Qualitative analysis of APP, MBP, and NF revealed no differences between injury groups, indicating no effect of mTBI on amyloid precursor protein, myelination, or neurofilament. Iba1 seemed to be more robust in the thalamus of 4RTBI compared with sham. For GFAP, the percent area stained was compared between injury groups in the grey-white matter junction, thalamus, and midline corpus callosum using an ANOVA.

For GFAP, there was a significant main effect of injury ($p=0.0324$) and time ($p=0.0256$) in the grey-white matter junction (Figure 19). Tukey post-hoc analysis revealed that GFAP burden was greater 1 week after 4RTBI (mean=9.79) than sham (mean=1.46). There was also a significant interaction effect of injury and time ($p=0.0260$; Figure 20).

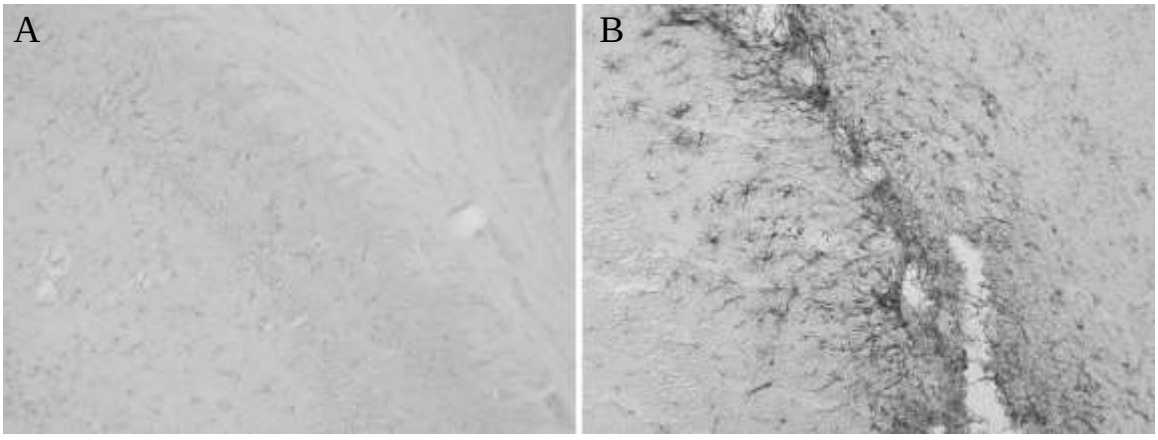

Figure 19. GFAP in the grey-white matter junction 1 week after A) sham and B) 4RTBI24

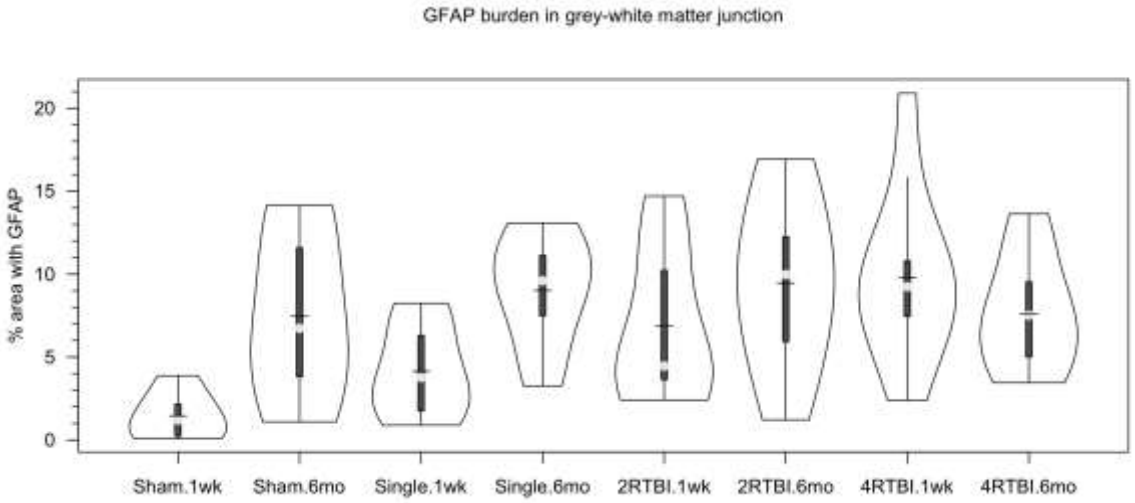

Figure 20. Violin plots representing percent area stained of GFAP 1 week and 6 months after sham, single TBI, 2RTBI, and 4RTBI in the grey-white matter junction. GFAP was significantly greater in 4RTBI versus sham.

For GFAP in the midline corpus callosum, there was a statistically significant main effect of time (p=0.0424) with more GFAP at the 6-month time point (mean=3.34) than at the 1-week time point (mean=1.37; Figure 21). There were no effects of injury (p=0.6946).

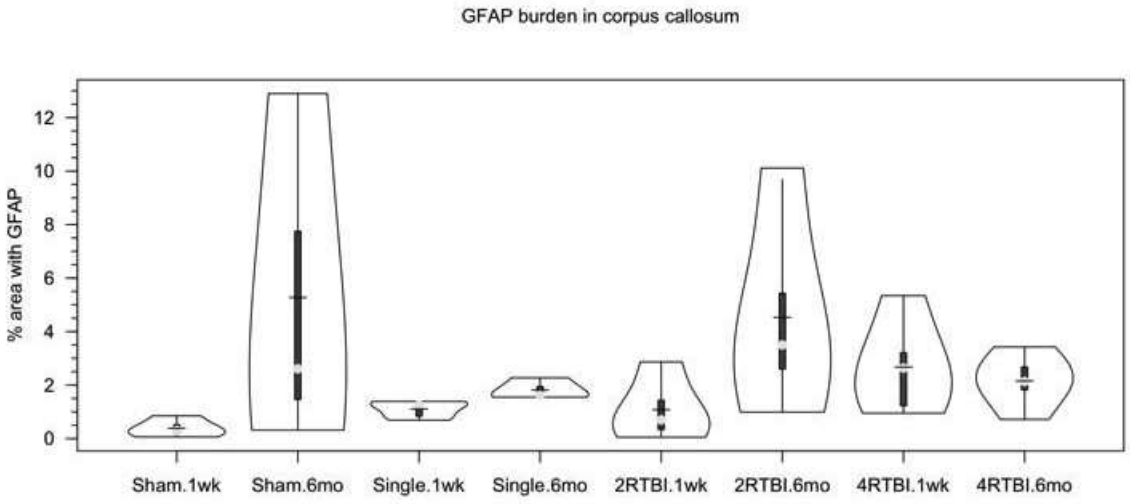

Figure 21. Violin plots representing percent area stained of GFAP 1 week and 6 months after sham, single TBI, 2RTBI, and 4RTBI in the corpus callosum. GFAP was significantly greater at 6 months compared to 1 week, but there was no injury effect.

In the thalamus (Figure 22), there was a significant main effect of injury ($p=0.0461$), but not time ($p=0.3772$). Tukey HSD post-hoc analysis revealed that GFAP burden was greater after 4RTBI (mean=4.82) than after sham (mean=2.25; Figure 23).

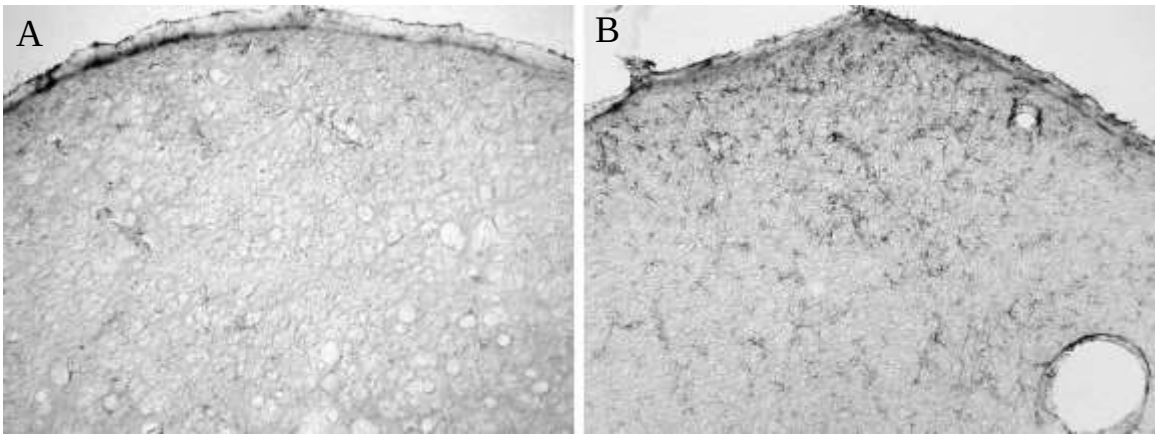

Figure 22. GFAP in the ipsilateral thalamus after A) sham and B) $4RTBI_{24}$

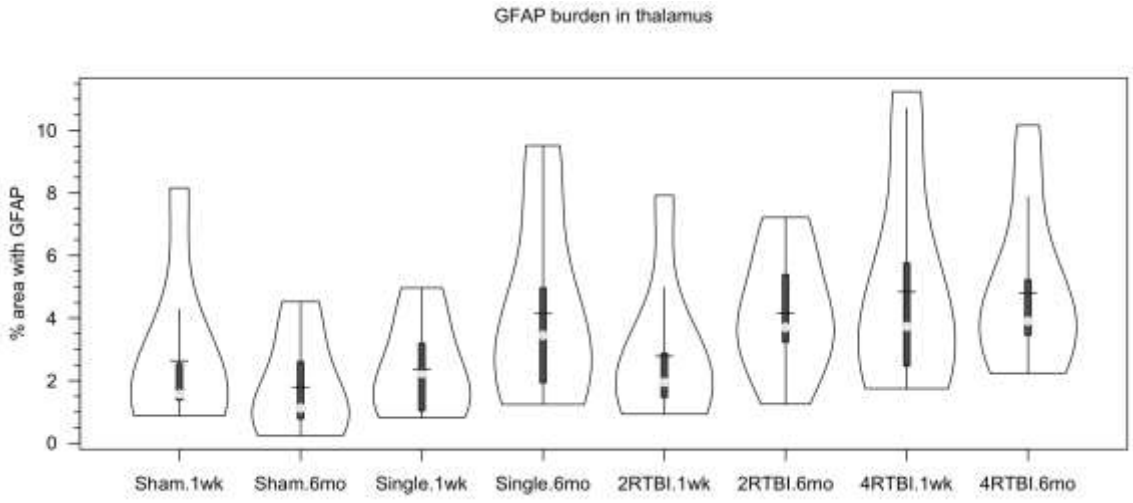

Figure 23. Violin plots representing percent area stained of GFAP 1 week and 6 months after sham, single TBI, 2RTBI, and 4RTBI in the thalamus. There was significantly more GFAP after 4RTBI than sham, with no time effect.

DISCUSSION

White matter changes after RTBI

Microstructural changes in white matter axonal bundles may occur after repeat mild traumatic brain injury (RTBI), serving as a mechanism by which RTBI can accelerate Alzheimer's pathogenesis. Conventional neuroimaging techniques, such as structural magnetic resonance imaging (MRI) and computed tomography (CT) are not sensitive enough to detect axonal injury (Rugg-Gunn et al., 2001; Arfanakis et al., 2002; Inglese et al., 2005). Diffusion tensor imaging (DTI), however, is an advanced neuroimaging technique that is able to calculate water diffusion properties in multiple directions to infer the orientation of white matter fiber bundles. This provides a non-invasive method of imaging subtle changes in the brain's microstructure, which may occur after mild TBI. Understanding the significance of these changes during the

recovery period after injury may provide researchers with insights in to the time profile of underlying pathology and possible mechanisms for AD-related protein accumulation. The effect of RTBI on white matter microstructure in the adolescent brain is unclear because the majority of DTI studies after human mTBI have focused on the adult brain. In addition, human studies also rely on self-reported concussion history, which is subject to recall bias. This animal study addressed both acute and chronic neuroanatomical changes following mTBI using tract-based spatial statistics (TBSS), a whole-brain analysis technique.

In this study of adolescent rats, white matter changed subacutely following RTBI. Fractional anisotropy (FA), or the degree to which water molecules diffuse along axonal bundles, serves as a proxy for white matter microstructure and is the most widely analyzed DTI metric. Omnibus statistical analysis with an F-test did not reveal any significant differences in FA between injury groups in the subacute time point of 1-week post-TBI. However, when a post-hoc analysis was run, significant voxels emerged, indicating lower FA 1 week post-4RTBI than after sham. Specifically, FA was significantly decreased in the genu and anterior body of the corpus callosum. In more posterior sections, significant voxels appeared in the ipsilateral corpus callosum, external capsule and fimbria. The post hoc analysis was performed despite the insignificant omnibus F-test because imaging statistics are one-sided, as is the case with TBSS, so it is possible that F-tests will not be significant, but pairwise t-tests will be, especially if the significance is only marginal. This discrepancy between the F-test and the post-hoc

pairwise comparison might also suggest that the DTI study was slightly underpowered, with a bigger sample size required to detect such subtle white matter changes.

Several adolescent human studies have been published after single mTBI. The acute findings in this study are in contrast to the majority of this human adolescent data, which shows an increase in FA in the corpus callosum (Wilde et al., 2008; Chu et al., 2010; Bazarian et al., 2012). Two other adolescent studies showed no change in FA after mTBI, however (Cubon et al., 2011; Maugans et al., 2012). The conflicting data is likely due to the diverse subject pool and the various experimental designs used in the human studies. For example, subjects were not discriminated based on the number of previous concussions. One study only included participants with a single TBI (Maugans et al., 2012), whereas others either did not report concussion history (Wilde et al., 2008; Chu et al., 2010) or had a mixed cohort of subjects with none, one or a few past concussions (Cubon et al., 2011; Bazarian et al., 2012). Furthermore, the time post-injury varied significantly from 24 hours to "less than one month" (Cubon et al., 2011), although the exact time was not specified. This wide range can have a significant effect on data interpretation since the direction and meaning of anisotropy changes after TBI changes over time (Mac Donald et al., 2007; Niogi and Mukherjee, 2010; Mayer et al., 2011; Eierud et al., 2014). Lastly, the mechanism of injury varied across and within subjects. One study had a combination of high-speed motor vehicle-related injury and low-speed sport-related injury (Wilde et al., 2008) and another only had 1 subject with a concussion, with the vast majority of subjects having experienced "subconcussive blows" (Cubon et

al., 2011). Controlling for the type of injury, concussion history, and time post-injury, while still aiming for statistical power, is challenging in human studies, but these limitations should affect global extrapolation of findings.

There is a lack of DTI studies after mTBI in adolescent animal studies, rendering exact comparisons difficult. Animal studies, such as this one, can better control for the number of injuries and time-post injury. Unfortunately, most animal studies are in adult animals using moderate to severe TBI models (Mac Donald et al., 2007). The acute results of this study are somewhat consistent with those in a clinical adult study, however, which found an increase in radial diffusivity (RD) in the cortex and corpus callosum acutely after RTBI (Arfanakis et al., 2002). Although RD is not the same measure as FA, they are often coupled, with decreases in FA corresponding with increases in RD and vice versa. These results are also similar to another study of six to eight-week old mice that showed no changes in FA one week after 2RTBI (Bennett et al., 2012). These mice only received 2 injuries spaced 24 hours apart, with the lack of anisotropy or diffusivity differences matching the 2RTBI24 cohort in this study. Unfortunately, the researchers did not assess changes after 4RTBI24 as was done in this experiment.

DTI was also acquired chronically after RTBI, with the results bolstering the sparse body of published literature. The 6-month data in this study revealed no differences in FA between any of the injury groups. The lack of white matter changes 6 months after RTBI was also seen in Mannix et al. (2013), but in adult mice. Since most symptoms resolve shortly after TBI, the long-term results were not surprising. However,

one study did find microstructural changes 60 days after RTBI (Donovan, 2014), but the injury model used was a moderate CCI, which involved a craniotomy. Chronic human DTI data is, in many ways, more difficult to attain due to loss of attrition. However, one study found no changes in FA 30 days after a single concussion in adolescents (Maugans et al., 2012). Alternately, college-aged football players (mean age = 22.5) demonstrated significantly increased FA in the corpus callosum and corticospinal tract 6 months after a concussion. Concussion history was not reported in this study (Henry et al., 2011).

Overall, even with the findings from this study, there seems to be a lack of consensus among the substantially limited adolescent literature. The inconsistencies among DTI data after mTBI may be due to the prevalence of self-reporting of concussion history, which is inherently biased. Furthermore, some of these studies are conducted on people who report to the emergency room after mTBI, which biases these cases as potentially more complicated mild. It is possible that some of these differences between studies may be attributed to variations in analysis methods. TBSS has already proven beneficial in many mTBI studies (Xiong, 2014; Fakhran, 2014; Messé, 2012; Cubon, 2011; Messé, 2011) and should replace manual drawing of ROIs or even VBM, at least until even better analysis methods are introduced. Lastly, these varied findings across studies may reflect the heterogeneity of TBI as a whole.

Pathological basis for DTI findings

The significance of these findings remains unclear, particularly since the meaning of DTI changes in general has yet to be determined. Primary DTI studies, without the confounding variable of TBI, have been conducted to better understand the implication of FA. In a study comparing mice with and without myelination, demyelinated mice had an FA value 20% less than the mice with myelinated axons (Song et al., 2002), suggesting that myelination contributes to FA. FA alone, however, cannot differentiate between axonal damage and demyelination (Boretius et al., 2012), hence the need for IHC analysis.

Immunohistochemistry has been used to provide a pathological basis for DTI findings after TBI, although only after moderate or severe injury. Overall, an increase in FA after TBI reflects gliosis, whereas a decrease in FA is associated with demyelination (Mac Donald et al., 2007; Budde et al., 2011). However, a study of mTBI suggested that axonal injury, instead of demyelination, contributes to decreased anisotropy (Kraus et al., 2007). In this study, immunohistochemistry was performed in the subacute phase (1 week post-TBI) and the chronic phase (6 months post-TBI). Sections were stained for amyloid precursor protein (APP), glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba1), and myelin basic protein (MBP).

Amyloid precursor protein (APP) has been shown within white matter tracts as a marker of axonal injury after TBI (Stone, 2002), but its immunoreactivity is dependent on the time-post injury. In this study, there was no APP labeling at either the 1 week or 6

month time point after RTBI. However, a previous study from our lab using this injury model demonstrated increased APP immunoreactivity in the grey-white matter junction 24 hours after 2RTBI (Prins et al., 2010), suggesting an initial, transient increase in axonal injury that subsides by 1 week post-RTBI. This finding is consistent with two previous studies of adult mice, which showed very sparse (Shitaka et al., 2011) or no APP staining between 24 hours and 7 days after two repeat closed-head mTBI injuries spaced 24 hours apart (Bennett et al., 2012). Acutely after a slightly more severe weight-drop injury in adult Sprague-Dawley rats, APP immunoreactivity significantly increased between 6 and 24 hours post-injury. By the 48-hour time point, there was a marked decrease in APP and by 10 days post-TBI, it was nearly nonexistent, only sparsely seen near the focal, hemorrhagic lesions (Stone et al., 2002), which are not characteristic of the current injury model. Axonal injury, using APP as a proxy, exhibits a post-RTBI time profile that increases within the first 24 hours before returning to sham levels. This finding is in contrast to the DTI results, which indicated a decrease in fractional anisotropy 1 week post-RTBI. This conflict between DTI and APP immunolabeling is consistent with a previous study of RTBI in adult mice (Bennett et al., 2012), which found changes in DTI despite negative APP findings. Interestingly, silver staining was better correlated with DTI results. Overall, these mismatched DTI and immunohistochemistry results may question the sensitivity of the histological APP assessment, which has traditionally been the norm for imaging axonal injury.

Astrocyte reactivity, as measured by GFAP, is consistently robust acutely and chronically after repeat mild TBI. In this study, GFAP in the grey-white matter junction was significantly greater after 4RTBI than sham 1 week after injury. Interestingly, GFAP in this region and in the corpus callosum was significantly greater across injury groups at the 6 month time point compared to 1 week. This may indicate that while astrocyte reactivity is greater after RTBI, it generally increases over time as well. Acute comparisons are limited, but GFAP does increase bilaterally after 2RTBI in the grey-white matter junction of the adolescent brain (Prins et al., 2013). These findings are consistent with the majority of long-term RTBI studies in adult animals as well (Mouzon et al., 2014; Mannix et al., 2013; Luo et al., 2014). One study showed that while GFAP increased acutely after RTBI in the cortex, hippocampus, and white matter, it returned to sham levels by 16 weeks post-injury (Uryu et al., 2002).

Markers of microglial activation and demyelination, Iba1 and myelin basic protein (MBP), respectively, have been used following moderate and severe experimental TBI, but are limited in the RTBI literature. In this study, there was not a qualitative difference with either marker between injury groups or across time. These findings are consistent with previous studies of mTBI. In an acute mTBI study with a closed-head injury model, electron microscopy showed evidence of demyelination in the corpus callosum 3 days after injury, which returned to baseline at 1 week (Mierzwa et al., 2015). Mannix and colleagues (2013) only examined Iba1-positive cells 6 months after RTBI, but did not measure a difference between injured and sham adult mice. Data was not

acquired earlier than 1 week in this study, so perhaps demyelination does occur more immediately after RTBI. Due to the mild nature of the injury, and based on these previous studies, it reasons that one marker of microglial activation and inflammation would subside by 6 months, post-injury, especially in accordance with the lack of DTI abnormalities.

Significance of traumatic axonal injury (TAI)

MTBI causes traumatic axonal injury (TAI) initiated by the force produced by the brain rapidly accelerating and decelerating (Kelley et al., 2007; Marmarou et al., 2005; Reeves et al., 2005). Interestingly, axon damage is not the result of shearing, as was initially thought (Povlishock et al., 1983). Instead, it is follows the secondary injury sequelae, such as bioenergetic failures and dysfunctional mitochondria (Okonkwo and Povlishock, 1999; Johnson et al., 2013). The TAI demonstrated in these DTI results holds significant implications for accelerated AD pathogenesis, since the initial damage disrupts axonal transport, forcing an accumulation of organelles and proteins, which leads to local axonal swelling (Arfanakis et al., 2002). After a moderate to severe CCI injury, damaged axons were discovered to be a major site of Aβ accumulation (Washington et al., 2014).

In addition to being a site for protein accumulation, which may be relevant to AD, TAI in the corpus callosum, in particular, may also have behavioral and symptom-related significance. The corpus callosum (CC) is particularly vulnerable to TAI following

mTBI (Parizel et al., 1998), as these findings support, with FA decreasing in the genu subacutely after mTBI in adults (Rutgers et al., 2008).

TAI after mTBI (Povlishock et al., 1983) can lead to generalized cognitive dysfunction (Povlishock et al., 1992), but due to its structure and function, damage to the corpus callosum may be particularly detrimental. The corpus callosum is the largest white matter fiber tract in the brain and connects the cerebral hemispheres (Clarke and Zaidel, 1994). The rostrum, genu, and anterior body of the corpus callosum connect the anterior hemispheres and the spenium and posterior body connect the posterior hemispheres (de Lacoste et al., 1985). The corpus callosum plays a significant role in cognition. Reduced integrity within this white matter tract is associated with impaired cognitive functioning in older adults (Voineskos et al., 2012). Furthermore, interhemispheric information processing speed is significantly impaired in the subacute phase after adult mTBI, which may be attributed to corpus callosum dysfunction. Specifically, visual reaction time is slower 4 weeks after mTBI than controls and is disproportionately affected when task difficulty increases (Mathias et al., 2004). Therefore, damage to the corpus callosum, even microstructurally, can have a significant impact, especially during development.

DTI as a biomarker for post-concussion symptoms

The ability to detect white matter changes after RTBI has translational value, with DTI indices correlating with post-concussion symptoms (PCS) and behavior. GCS has

been positively correlated with FA (Yuan, 2007) and negatively correlated with ADC, also referred to as MD (Wilde, 2010). When assessed with self-reported symptom questionnaires within a week of mTBI in adolescents, PCS severity was correlated with increased FA and decreased RD in the corpus callosum (Wilde, 2008). In an overlapping, but slightly later time period after mTBI, decreased mean trace and increased FA in the internal capsule and corpus callosum correlated with PCS (Bazarian 2007). One month after mTBI, postconcussion symptoms were associated with decreased FA in the inferior fronto-occipital fasciculus (IFO), internal capsule, and the corpus callosum. Additionally, increased MD correlated with more severe PCS in the IFO, inferior longitudinal and superior longitudinal fasciculi (Smits, 2011). Behaviorally, higher FA correlates with faster cognitive processing and interference resolution (Wilde, 2006), suggesting that decreases in FA after TBI may have a behavior implication. Furthermore, microstructural alterations may persist beyond resolution of neurocognitive impairments. TBSS analysis of ice hockey players revealed increases in FA, but no differences in neuropsychological testing or symptoms, using ImPACT or SCAT2, respectively (Sasaki 2014). Although the cost and accessibility of DTI prevent it from being used as a sideline diagnostic measure, the correlation between DTI metrics and post-concussion symptoms or behavior may help elucidate the microstructural basis for neurocognitive impairment.

Limitations and future studies

One general limitation with DTI is that its metrics infer changes at the microscopic level, but DTI has relatively poor spatial resolution, assessing voxels that are 4-6mm^2. Until researchers know the precise meaning of DTI metrics, results should be interpreted with caution. In the future, the best modality may be multimodal imaging that combines the strengths of diffusion tensor imaging and functional MRI. A combined fMRI/DTI study of mTBI revealed that a reduced BOLD response in the inferior and medial frontal gyrus, areas involved in working memory, corresponded with decreased FA in the corpus callosum (Dean et al., 2015). This demonstrates the potential far reaching effect of DAI in the corpus callosum and suggests the utility of multimodal imaging to provide a more complete understand of the shared characteristic of white matter damage after mTBI and in AD.

Conclusions

This study was the first to examine both acute and chronic DTI changes after RTBI in the adolescent rat. Concussion rates have steadily increased among adolescents, an age characterized by robust brain development, which may make the brain during this period particularly vulnerable to the consequences of injury. FA increases during adolescence, which likely reflects robust myelination of axons (Lebel et al., 2012). Perturbations during this period, then, may be particularly disruptive, so it is important to understand if interruptions in normal white matter development can effect long-term

impairment. The decreases in anisotropy subacutely after RTBI were not paralleled by measures of immunohistochemical axonal injury or demyelination. This mismatch challenges the sensitivity of traditional immunohistochemical techniques and probes a better understanding of the neuropathological significance underlying DTI indices.

CHAPTER 3.

TO DETERMINE THE ACUTE EXPRESSION PROFILES OF AD-RELATED PROTEINS FOLLOWING RTBI IN THE ADOLESCENT RAT

INTRODUCTION

Repeat mild TBI (RTBI) in the adolescent brain may accelerate amyloidogenesis later in life, but the mechanism by which this occurs is unknown. One possibility is that RTBI causes axonal damage, as was demonstrated in Chapter 2, which leads to protein transport disruption and an accumulation of proteins, including those involved in amyloid beta (Aβ) production (Chen et al., 2009). Aβ is formed by sequential cleavage of the transmembrane protein amyloid precursor protein (APP) by two enzymes, BACE1 and PS1 (De Strooper et al., 1998). Once Aβ is cleaved from APP, it can bind to other Aβ monomers to create dimers or trimers (Hardy and Higgins, 1992). These dimers and trimers can continue to aggregate, forming toxic, soluble oligomers. These Aβ oligomers are soluble and thought to more strongly correlate with cognitive impairment than the insoluble, aggregated Aβ plaques (McLean et al., 1999). Furthermore, these oligomers can increase Ca^{2+}, hyperphosphorylate tau, and cause synaptic dysfunction (Zempel et al., 2010). Aβ accumulation is known to play a role in the pathogenesis of Alzheimer's disease, so proteins involved with inducing the amyloidogenic pathway after injury may help explain a correlation between TBI and AD.

There is a clear association between TBI and AD-related proteins, but this finding has only been established after moderate to severe TBI. In humans, BACE1 and PS1 increase up to 4 weeks after a severe TBI (Uryu et al., 2007). This pattern is similar in animal models with both BACE1 and PS1 increasing acutely after moderate CCI (Blasko et al., 2004; Loane et al., 2009; Washington et al., 2014) or a moderate closed-head injury (Nadler et al., 2008). Furthermore, BACE1 increases after cerebral metabolic depression (Struble et al., 2010), which has been shown in this injury model and is exacerbated with decreased BII (Prins et al., 2013). Since the enzymes required to produce Aβ increase after TBI, it reasons to follow that Aβ itself would also increase after injury. In humans, soluble $A\beta_{42}$ elevation persists for 5 weeks after injury, although time points beyond that were impossible to measure because patients succumbed to their injuries. In transgenic mice, soluble Aβ does indeed increase in the hippocampus and cortex up to 48 hours post-TBI (Smith et al., 1998; Tran et al., 2011; Washington et al., 2014), before returning to baseline by 72 hours (Washington et al., 2014). The post-RTBI temporal profile of the APP cleavage enzymes, BACE1 and PS1, as well as the soluble Aβ product may provide insight in to the mechanisms underlying chronic amyloidogenesis after RTBI.

The aim of this study was to measure the changes in AD-related proteins (i.e. soluble Aβ, BACE1 and PS1) in the cortex and hippocampus acutely after 4RTBI. Triple transgenic Alzheimer's rats (3xTg-AD) were used to determine Aβ oligomer concentration. Wild-type Sprague-Dawley rats were used for the secretase analysis due to the 3xTg-AD rats overexpressing PS1, rendering changes in the saturated concentrations

undetectable. We hypothesized that BACE1, PS1, and Aβ oligomers would increase in the acute time period following 4RTBI.

METHODS

Experiment 1.

Experimental design and subjects

Postnatal day 35 (PND35) male Sprague-Dawley rats received sham or quadruple injuries spaced 24 hours apart ($4RTBI_{24}$). They were sacrificed for immunohistochemistry either 24 hours or 48 hours after the last injury (Figure 24). The number of rats per group was as follows: sham (n=8), $4RTBI_{24}$ at 24 hours (n=6), 4RTBI at 48 hours (n=6). All procedures were approved by the UCLA Chancellor's Committee for Animal Research

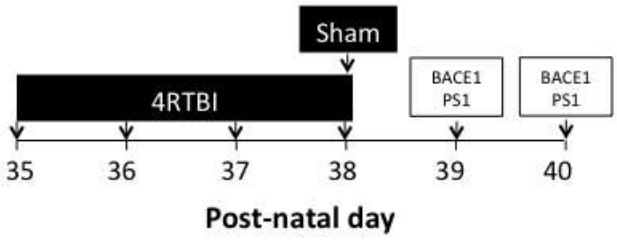

Figure 24. Experimental design aim 3a

Closed-head injury model

Same as in Chapter 1

Tissue preparation

The ipsilateral cortex was dissected, flash frozen in methylbutane on dry ice, and stored in -80°C.

Western blot for BACE1 and PS1

Tissue was homogenized in RIPA buffer containing 1% Triton X-100, 1mM EDTA, 0.5% NP-40, 150 mM NaCl, and 10mM Tris. Samples were run on 4-20% Tris-HCL gel for the PS1 antibody and 7.5% Tris-HCL Criterion gel for the BACE1 antibody. After transfer, membranes were incubated in 7% Acetic/10% MetOH for 15 minutes, washed in ddH_2O, and incubated in Syrpo Ruby for an additional 15 minutes. The membrane was imaged with the Quantity One computer imaging software. The membrane was blocked with 5% milk – TTBS for 1 hour and then incubated overnight in a 1:1000 (BACE1) or 1:500 (PS1) primary antibody – TTBS solution at 4°C. On day 2, the membrane was washed and then incubated for 1 hour in secondary antibody (anti-mouse for PS1; anti-rabbit for BACE1) in 1% milk-TTBS. After four TTBS washes, the membrane was covered with ECL and imaged. Protein levels were normalized to the

ruby stain and analyzed densitometrically with Image J (National Institutes of Health) software.

Experiment 2.

Experimental design and subjects

Postnatal day 35 (PND35) male and female triple transgenic rats (3xTgAD) received sham or quadruple injuries spaced 24 hours apart ($sham_{24}$ or $4RTBI_{24}$), or sham or quadruple injuries spaced 72 hours apart ($sham_{72}$ or $4RTBI_{24}$; Figure 25). The number of rats per group was as follows: male $sham_{24}$ (n=3), male $4RTBI_{24}$ (n=4), male $sham_{72}$ (n=3), male $4RTBI_{72}$ (n=4), female $sham_{24}$ (n=4), female $4RTBI_{24}$ (n=4), female $sham_{72}$ (n=4), female $4RTBI_{72}$ (n=4). Rats were sacrificed for immunohistochemistry 24 hours after the last injury. All procedures were approved by the UCLA Chancellor's Committee for Animal Research.

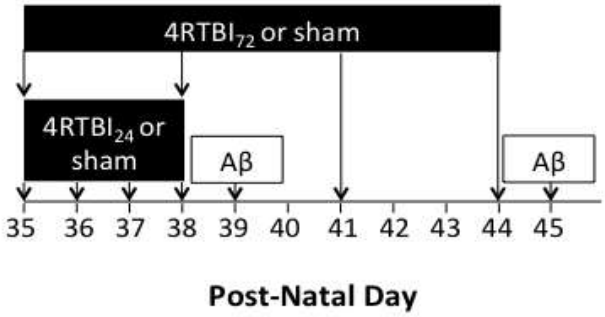

Figure 25. Experimental design aim 3b

Closed-head injury model

Same as in Chapter 1

Tissue preparation

The ipsilateral hippocampus was dissected, flash frozen in methylbutane on dry ice, and stored in -80°C.

Oligomeric Aβ Dot Blot

Soluble, conformational-dependent, oligomeric Aβ species in the hippocampus were analyzed by dot blot assays a using an anti-prefibrillar oligomer (A11) amyloid antibody, based on a previously described protocol (Kayed, 2003). Rat brain extracts (8 ug of total protein per well; the total protein concentration was measured using Pierce

BCA Protein Assay Kit according to the manufacturer's protocol) were applied in duplicates to the rehydrated nitrocellulose membrane using Bio-Dot microfiltration apparatus (Bio-Rad). After 2 washes with Tris-buffered saline (TBS), membrane was removed from the Bio-Dot apparatus, blocked with 10% fat free dry milk in TBS with 0.01% Tween 20 (TBS-T) for 1hr at room temperature and probe with A11 (1:1000) diluted in TBS-T with 5% fat free dry milk overnight at 4°C. The membrane was washed 4 times with TBS-T and incubated with HRP-conjugated secondary antibody for 1hr at room temperature followed by 3 washes with TBS-T and 1 wash with TBS. Chemiluminescent signals were detected with Super Signal West Femto substrate (Pierce Chemicon) using UVP BioSpectrum 600 imaging system (Upland, CA) and VisionWorks software.

Total oligomeric $A\beta_{42}$ ELISA

Tissue from $sham_{24}$ and $4RTBI_{24}$ was homogenized. Soluble, total oligomeric $A\beta_{42}$ was analyzed using a sandwich ELISA with the same monoclonal anti-$A\beta_{42}$ antibody (10G4; Mak et al., 1994; Yang et al., 1994) as both the capture and the reporter. High-bind 96-wells ELISA plates were coated with unlabeled 10G4 antibody in 0.1 M carbonate buffer, pH 9.6 [overnight (ON) at 4°C] followed by blocking with 4% BSA in PBS [(4 hr at room temperature (RT)]. A 7-point standard curve (range from 1.95 to 125 ng/ml) was constructed using photochemically cross-linked synthetic $A\beta_{42}$ oligomers. Samples and standards were diluted in 1% BSA in PBS and loaded into plates in

duplicates (ON at 4°C). After washing with PBS, biotinylated 10G4 antibody was applied (ON at 4°C). Then, plates were washed with PBS and incubated with streptavidin-HRP (1 hr at RT). The immunocomplex was reacted with TMB (3,3',5,5'-tetramethylbenzidine) substrate and detected using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). Concentrations of $A\beta_{42}$ oligomers in samples were calculated from the standard curve.

Statistical analysis

All statistical analyses were performed using R software (version 3.0.0). Analysis of variance (ANOVA) with Tukey-Kramer post-hoc was used to compare normalized optical densities of BACE1, PS1, and soluble Aβ oligomers, as well as concentration of total Aβ.

RESULTS

Experiment 1.

There were no significant differences in BACE1 optical density between sham or $4RTBI_{24}$ 24 or 48 hours after injury (F=0.0, df=1, p=0.9963). For PS1, there was a significant main effect for injury (F=5.357, df=1, p=0.032), but not time (F=2.366, df=1, p=0.140), with optical density for 4RTBI (mean=1.17) significantly less than sham (mean=1.72) across both times points (Figure 26).

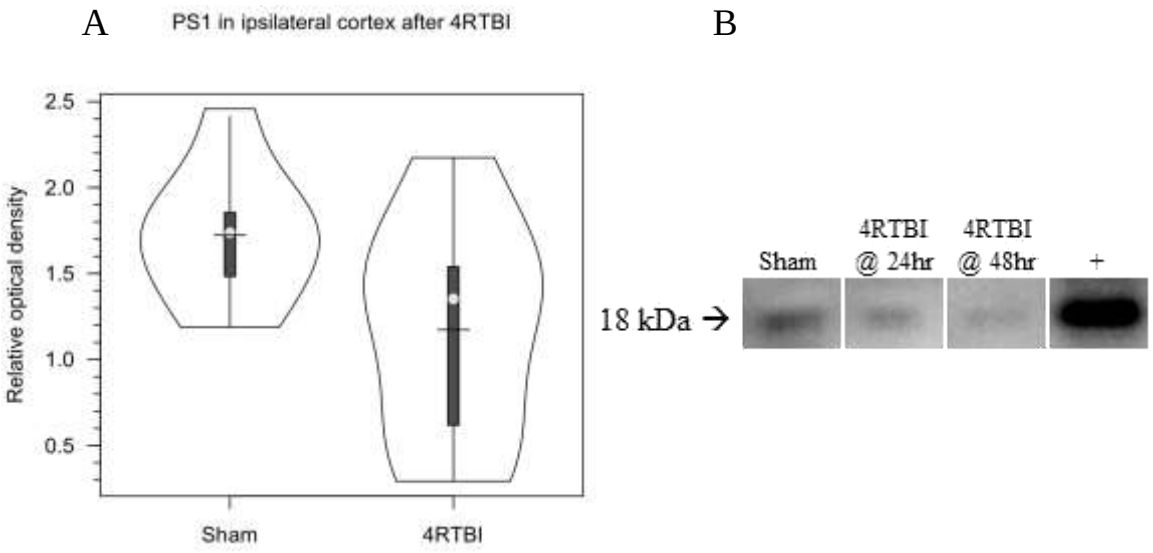

Figure 26. PS1 in the ipsilateral cortex after sham and 4RTBI. A) The violin plot represents a main effect for injury, but not for time post-4RTBI. B) Western blots of PS1 after sham, 24 hours post-4RTBI, and 48 hours post-4RTBI, and positive control (from 3xTg-AD that overexpresses PS1)

Experiment 2.

For the soluble, Aβ oligomers (Figure 27), there was no statistical difference between males and females (p=0.2566), so the genders were pooled. There was a

significant main effect of both injury (F=7.470, df=1, p=0.011) and interval (F=4.837, df=1, p=0.0369). There was 35.17% more soluble Aβ after 4RTBI than sham and 28.9% more with an injury interval of 72 hours versus 24 hours. Preliminary data for total $A\beta_{42}$ indicated no statistical difference between males and females (p=0.1070), so the genders were pooled. There was a significant difference between sham (mean=0.32) and $4RTBI_{24}$ (mean=0.57) 24 hours after injury (F=9.625, df=1, p=0.0112).

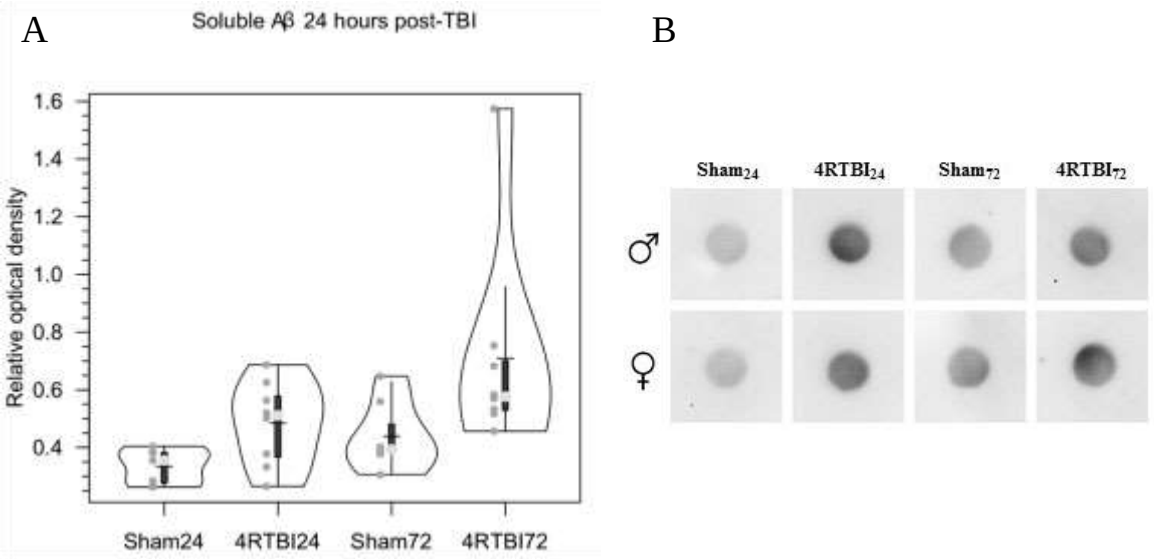

Figure 27. Soluble Aβ in the ipsilateral hippocampus 24 hours post-injury. A) Violin plot of the relative optical density of soluble Aβ in the ipsilateral hippocampus. This plot represents a main effect for injury and BII. There was no gender effect. B) Dot blots of soluble Aβ in male and female 3xTg-AD rats.

DISCUSSION

The acute effect of RTBI on secretases

Acute disruption of axonal bundles after RTBI may lead to an accumulation of AD-related proteins, providing a mechanism by which RTBI can exacerbate the deposition of Aβ. Two of these AD-related proteins, BACE1 and PS1, sequentially cleave amyloid precursor protein (APP) to form Aβ monomers of various lengths (De Strooper et al., 1998; Selkoe and Wolfe, 2000). These monomers self-bind to form neurotoxic oligomers. BACE1 is often referred to as the rate-limiting step in Aβ production, which provides an important site of exploration after RTBI. In this study, there were no changes in BACE1 concentration either 24 or 48 hours after 4RTBI. There have not been any studies measuring changes in BACE1 after mild TBI, much less in the adolescent brain, so comparisons can only be drawn with studies utilizing more severe injuries in adults, such as the CCI model. The moderate and severe TBI literature is consistent, but contrary to the results of this study, showing an increase in BACE1 up to 3 days after TBI before returning to baseline by 7 days (Blasko et al., 2004; Loane et al., 2009; Washington et al., 20140). It is not surprising that different injury severities produce different BACE1 profiles acutely after the insult. Furthermore, BACE1 concentration may indeed increase acutely, but return to baseline by 24 hours post-RTBI.

The second secretase required for APP cleavage and Aβ production is presenilin-1 (PS1). After RTBI, PS1 was significantly less compared to sham. The time post-injury, either 24 or 48 hours, was not associated with this decrease, however, indicating that the

difference in PS1 concentration from 24 to 48 hours is not significant. This finding is in contrast to another closed-head injury study in adult mice (Nadler et al., 2008). The ipsilateral cortex and hippocampus were stained for immunoreactivity and showed an increase in PS1 beginning 48 hours after a modified weight-drop injury. This increase, compared to sham, peaked 4 to 7 days after mTBI before gradually declining. This discrepancy is likely due to variances of injury severity. Although both models are classified as mild, the weight-drop model produces overt cell death, particularly in the hippocampus (Chen et al., 1999), which is not seen in the current model (Prins et al., 2010).

Soluble Aβ post-RTBI

Aβ aggregates in several distinct, yet fluid, ways, including in to soluble monomers, pre-fibrils, fibrils, insoluble Aβ plaques, and an infinite number of intermediate confirmations. To gain a better understanding of the acute effect of RTBI on some of these various forms of Aβ, we measured confirmation-specific, pre-fibrillar Aβ 24 hours after $4RTBI_{24}$ and $4RTBI_{72}$. Despite the negative finding of BACE1 after TBI, and the observation that PS1 actually decreases 24 and 48 hours after injury, we needed to examine whether or not soluble Aβ still increases. Since wild-type rats do not produce ample Aβ, 3xTg-AD rats were used for these Aβ experiments, as opposed to Sprague-Dawleys for the previous enzyme experiments.

In this study, soluble Aβ, as measured by the A11 antibody, was affected by RTBI acutely. This pre-fibrillar form of Aβ was significantly greater 24 hours after 4RTBI than sham, with a statistical difference between the $4RTBI_{24}$ and $4RTBI_{72}$ groups. However, the violin plot indicates an outlier in the $4RTBI_{72}$ group, which may be skewing the data. Close examination of the subject's physiological data and the tissue preparation did not indicate any abnormalities, so there was no scientific justification for removing this outlier. Additionally, preliminary total $A\beta_{42}$ analysis, which has often been shown as the most toxic Aβ species (Goure et al., 2014) revealed increased concentration 24 hours after $4RTBI_{24}$. This preliminary finding is consistent with previous work (Smith et al., 1998; Tran et al., 2011; Washington et al., 2014). The nearly unlimited array of Aβ forms, each with a potentially different role, justifies the need to utilize antibodies against these various forms, especially if they have not been studied in the TBI context.

This data demonstrates that 4RTBI has a robust acute effect on oligomeric Aβ concentration irrespective of the varied BII, with both $4RTBI_{24}$ and $4RTBI_{72}$ showing increases relative to sham. Interestingly, the acceleration of Aβ plaques at the chronic time point was only present after $4RTBI_{24}$, when the BII was 24 hours. Why did the $4RTBI_{72}$ group, which also showed elevated Aβ oligomers acutely after 4RTBI, not demonstrate accelerated chronic Aβ pathology? The answer might lie within the effect of BII on cerebral metabolic rate of glucose (CMRglc), as has been previously demonstrated in 2DG studies with this injury model. CMRglc is depressed after a single mild injury. This depression is greater in magnitude and duration when a subsequent injury occurs

during this period of vulnerability, prior to CMRglc recovery (as is the case with a BII of 24 hours). Hypometabolism is an early cellular event in AD, preceding clinical symptoms, so this early perturbation in glucose metabolism might contribute to triggering the disease process. The repeat injuries in the $4RTBI_{72}$ group occurred after the brain metabolically recovered, perhaps protecting it from hypometabolism-induced pathological sequelae. This data might suggest the critical role of post-injury glucose metabolic depression in long-term pathological consequences.

Potential mechanisms underlying soluble Aβ increase

How can soluble Aβ increase without a parallel increase in the enzymes that produce Aβ? One reason may be based on the different animal models used in this study. For the reasons previously discussed, Sprague-Dawley rats were used for the acute enzyme experiments and 3xTg-AD rats were used for the soluble Aβ experiments and the chronic Aβ accumulation work. It is possible that with this injury model, changes in the secretases as measured in the Sprague-Dawley rats might not reflect the acute or chronic sequelae in the 3xTg-AD rats. We know that the baseline concentrations between these animal models are different since the 3xTg-AD rats overexpress PS1. However, a preliminary study of BACE1 western blots in the 3xTg-AD rats did not show differences between sham and injured groups either. Therefore, if this Sprague-Dawley data is indeed reflective of the 3xTg-AD rats and, in both models, BACE1 does not increase 24

or 48-hours after injury, then secretase concentration alone cannot directly explain the long-term increase in Aβ plaque accumulation.

Another possible explanation for the increase in Aβ without a corresponding increase in BACE1 concentration is that the concentration of an enzyme does not necessarily dictate the activity of that enzyme. Specifically, TBI might not increase the amount of BACE1, but it might exacerbate the enzymatic activity of BACE1. One marker of BACE1 activity is β-CTF, the C-terminal fragment byproduct of BACE1 cleavage of APP. β-CTF has been shown to increase 1 day and 3 hours after a moderate or severe CCI before returning to baseline by 7 days (Washington et al., 2014). However, preliminary and unpublished work in our lab demonstrates that β-CTF does not increase 24 hours after 4RTBI, again suggesting a different secretase response after a milder, closed-head injury. Furthermore, preliminary results did not reveal changes in α-CTF, the C-terminal fragment byproduct of α-secretase, which would indicate a shift in APP processing toward the non-amyloidogenic pathway.

Another possible reason for this uncoupling is that BACE1 concentration remains unchanged (or increases transiently), so the production rate of Aβ remains steady, but the Aβ clearance mechanism is impaired. Several Aβ clearance pathways have been studied in AD as an explanation for Aβ accumulation in sporadic AD (Baranello et al., 2015). Of particular relevance is the glymphatic system, a recently discovered waste clearance system in the brain. Although this system was first described in 2012 by Iliff and colleagues, it is just beginning to garner attention. The glymphatic system is essentially

the cerebral lymphatic system. Instead of traditional lymphatic vessels to clear soluble waste, the glymphatic system utilizes the continuous exchange between interstitial fluid (ISF) and cerebrospinal fluid (CSF) to rid the brain of protein waste (Jessen et al., 2015). Interestingly, the dynamic CSF-ISF interchange and the glymphatic system as a whole is increasingly impaired with age (Kress et al., 2014). Furthermore, the glymphatic system is enabled by aquaporin-4 (AQP4)-mediated water channels, which are impaired and mislocalized after TBI (Ren et al., 2013). Most importantly, as it relates to this study, clearance of soluble Aβ requires a functional glymphatic system (Iliff et al., 2012). So, might the glymphatic system be impaired after TBI as well, potentially explaining the increase in soluble Aβ independent of increased secretase concentration? A recent study demonstrated that a moderate TBI impairs glymphatic pathways and increases phosphorylated tau in the ipsilateral cortex (Iliff et al., 2014).

Overall, 4RTBI does not change secretase concentration (or activity, as examined in preliminary experiments) in Sprague-Dawley rats, but it does increase oligomeric Aβ acutely, independent of the BII, in 3xTg-AD rats. BII does have an effect on chronic insoluble Aβ burden, however, which perhaps suggests differences in the Aβ clearance mechanisms and/or the influence of the duration of hypometabolism. These two questions are important areas of research moving forward.

Significance of soluble Aβ oligomers increasing after RTBI

The increase of soluble Aβ oligomers poses a significant risk to various pathological processes. Soluble Aβ, particularly in the cortex and hippocampus, is strongly correlated with cognitive impairment in AD patients (McClean et al., 1999; Lue et al., 1999). The mechanism for this interaction is not completely understood, but it likely centers on the relationship between soluble Aβ (also known as Aβ-derived diffusible ligands (ADDLs)) and synapses. ADDLs, as were examined in this study, seem to initiate synaptic dysfunction, leading to cognitive impairment (Selkoe, 2008). This neurotoxic characteristic of ADDLs is in contrast to the perhaps innocuous Aβ monomers and fibrils (Giuffrida et al., 2009). One potential binding site for Aβ oligomers in transgenic mice is the paired immunoglobulin-like receptor B (PirB). Mice without these receptors do not develop Aβ-induced attenuations of long-term potentiation (LTP) or memory deficits (Kim et al., 2013).

Other toxic consequences of increased soluble Aβ involve glutamate, LTP, tau, apoptosis, and prion-like pathways. Oligomers have been shown to upregulate glutamate, which can overstimulate NMDA receptors, eventually leading to mitochondrial dysfunction and synaptic loss (Hamilton et al., 2015). Dimers and trimers have been shown to inhibit hippocampal LTP (Shankar et al., 2007; Shankar et al., 2008; Walsh et al., 2002). Aβ oligomers can also lead to increases in hyperphosphorylated tau (De Felice et al., 2008; Tomiyama et al., 2010; Zempel et al., 2010), which can instigate tau aggregation, the second pathological hallmark of Alzheimer's disease. Soluble oligomers

have also been shown to induce apoptosis directly, by binding to the p75 neurotrophin receptor and activating its pro-apoptotic domain (Yaar et al., 1997). Lastly, soluble Aβ can self-propogate in a prion-like mechanism, whereby seed deposits of Aβ can replicate and spread, enhancing its own propagation (Kumar et al., 2014). Clearly, an increase in soluble Aβ likely damages synapses, impairing synaptic function and potentially initiating memory deficits.

Limitations and future studies

This study does have a few limitations that can be addressed with future studies. One limitation of this study in the greater context of this dissertation is that we did not look extensively at BACE1 or PS1 in the transgenic model. Since these 3xTg-AD rats overexpress PS1, it is impossible to measure changes in PS1, since concentrations reach saturation. Regarding BACE1, the priority for the limited 3xTg-AD rats were the chronic, 12-month studies described in chapter 1. However, preliminary results did not show a difference in BACE1 between injured and sham 3xTg-AD rats. Since soluble and total Aβ increases after RTBI, it is feasible that BACE1 also increases after RTBI, but perhaps the time profile is much shorter than that after a more severe injury. Future studies could measure secretases during the sub-24 hour phase, at 6 hours or 1 day, for example, when increases are initially seen after CCI (Blasko et al., 2004; Loane et al., 2009). Lastly, in relation to the secretases, the western blot data in this study only

reflects changes in enzyme concentrations. Future studies should examine both enzymatic concentrations and activity after injury.

Ultimately, it is important to examine the relationship between TBI and AD as a whole and consider the other pathological hallmark of the disease, neurofibrillary tangles (NFTs). Previous studies have cited an increase in both Aβ and tau after moderate and severe TBI (Tran et al., 2011). However, as was discussed in chapter 1, this question could not be addressed with this animal model, which does not produce enough hyperphosphorylated tau to aggregate in to NFTs. While we chose to look at two oligomeric Aβ species, one that has been studied extensively after TBI and another that has not been examined, there are an exorbitant number of additional oligomeric species that could be assessed, each of which may have a different neurotoxic contribution. Aβ peptides dynamically move between conformations and at any given time can occupy the shape of an α-helix, a β-sheet, or an indistinguishable coil (Kirkitadze et al., 2001; Miyashita et al., 2009). These various structures then combine to produce oligomers of different toxicities (Ono et al., 2009) and should be measured after TBI.

Lastly, due to the limited number of 3xTg-AD rats available for biochemistry, we were unable to assess soluble Aβ at multiple time points after injury. Future studies should develop a time profile of this protein after both $4RTBI_{24}$ and $4RTBI_{72}$ to determine how long these increases in soluble Aβ persist. Previous studies suggest that $A\beta_{42}$ can remain elevated up to 3 weeks after CCI (Abrahamson et al., 2006) and returns

to baseline by 6 months post-RTBI (Mannix et al., 2013). This pattern has not been established in the adolescent brain or with BII as a factor, however.

Conclusions

This aim examined acute changes in AD-related proteins after RTBI that may influence long-term Aβ accumulation. The long gap between RTBI in adolescence and Aβ plaque formation needs to be studied and this inquiry should begin in the acute phase after injury. Biochemical analysis of the cortex and hippocampus, two regions particularly susceptible to Aβ deposits, measured the BACE1 and PS1 secretases, as well as Aβ oligomers, acutely after RTBI. Aβ oligomers increased 24 hours and 48 hours after injury, despite no changes in BACE1 concentration and a decrease in PS1, which may be due to impaired clearance mechanisms. Increased Aβ oligomer concentration is of particular concern, given the many synaptic dysfunction and apoptotic pathways they can initiate. There are clearly acute changes in AD-related pathophysiological molecules that may trigger pathways implicated in amyloidogenesis.

DISCUSSION OF THE DISSERTATION

SUMMARY OF FINDINGS

The results of this study significantly contribute to our understanding of the complex relationship between traumatic brain injury (TBI) and Alzheimer's disease (AD). Previous studies have centered on the adult brain, but with the incidence of repeat mild concussions higher in adolescence, focus on this age group is warranted. Moderate and severe TBI are robust risk factors for AD, but the effect of repeat mild traumatic brain injury (RTBI) and the brain impact interval (BII) in adolescence has not yet been determined.

In chapter 1, we established a positive correlation between repeat mTBI (RTBI) and accelerated Aβ, the main pathological marker of AD. Postnatal day 35 triple transgenic male and female Alzheimer's rats (3xTg-AD) received no injuries (sham), 4 injuries spaced 24 hours apart ($4RTBI_{24}$) or 4 injuries spaced 72 hours apart ($4RTBI_{72}$), with subsequent injuries occurring after the cerebral metabolic rate of glucose (CMRglc) returns to sham levels (Prins et al., 2013). Approximately 10.5 months after injury, when the rats were 12 months of age, immunohistochemical analysis of Aβ revealed a significant increase in the percent area occupied by plaques in the hippocampus after $4RTBI_{24}$ that did not vary with gender. Particularly noteworthy is the fact that this increase was reversed when the BII was increased, after $4RTBI_{72}$. Qualitative immunohistochemical analysis showed an increase in astrocyte reactivity (as measured

by GFAP staining) 24 hours after $4RTBI_{24}$, especially in the molecular layer, which seemed to subside by 1 week and 8 months post-RTBI. Inflammation seemed to persist longer after injury, with microglia activation increased in the ipsilateral hippocampus up to 1 week post-$4RTBI_{24}$. Ultimately, this chapter shows that RTBI is correlated with chronic elevated Aβ burden in transgenic rodents in an injury-interval related manner.

The primary question that emerged from chapter 1 is central in the TBI-AD literature: What is the mechanism by which an early injury causes an accelerated disease response? It is highly improbable that RTBI results in an increase of a single molecule or a protein that persists over the course of months (or decades in humans), leading to AD pathology. Instead, TBI-induced changes in several processes likely trigger additional events that combine with normal aging to accelerate the deposition of Aβ plaques. One early event after TBI is traumatic axonal injury (TAI). Axonal breakage is believed to contribute to an accumulation of AD-related proteins, which may then trigger the disease process (Chen et al., 2004). The extent of TAI acutely after RTBI in the adolescent brain has not been established. In aim 2, we used diffusion tensor imaging (DTI), an advanced MRI technique sensitive enough to detect axonal injury after TBI (Rugg-Gunn et al., 2001; Inglese et al., 2005). One week after $4RTBI_{24}$, fractional anisotropy, a measure of the direction of water molecules, decreased in the genu and anterior body of the corpus callosum as well as the external capsule. FA was not significantly altered after either a single or double mTBI, however. By the 6-month time point, FA returned to baseline and was not significantly different across injury groups. Corresponding

immunohistochemistry against amyloid precursor protein (a marker of axonal injury), myelin basic protein (a marker of demyelination) and Iba1 (a marker of inflammation) did not reveal any differences between injury groups at either 1 week or 6 months post-TBI. However, astrocyte reactivity (as measured by GFAP) was significantly greater in the grey-white matter junction after $4RTBI_{24}$ than sham at the 1-week time point. Overall, DTI detected traumatic axonal injury in the corpus callosum and external capsule 1 week after $4RTBI_{24}$ that was not identified through immunohistochemistry.

The aim of chapter 3 was to identify early changes in proteins after RTBI that are known to be involved in AD pathogenesis and may accumulate in damaged axons. BACE1 and PS1, the cleavage enzymes required to produce Aβ from APP (De Strooper et al., 1998; Vassar et al., 1999; Selkoe and Wolfe, 2000), are known to increase after a single moderate or severe TBI and RTBI (Uryu et al., 2007; Washington et al., 2014). Additionally, the product of this sequential cleavage, Aβ monomers, binds to form toxic Aβ oligomers and post-traumatic changes in these oligomers may also contribute to long-term pathogenesis. Somewhat surprisingly, neither BACE1 nor PS1 increased after TBI in the Sprague-Dawley rats (in fact, PS1 decreased slightly). However, the concentration of Aβ oligomers was elevated 24 hours and 48 hours after 4RTBI in the 3xTg-AD rats. These results indicate that RTBI may increase Aβ oligomers without a corresponding increase in the cleavage enzymes. Future research should investigate the efficiency of Aβ clearance mechanisms after RTBI as a potential explanation for the accumulation of Aβ oligomers.

Taken together, these findings suggest an association between closely spaced repetitive adolescent mTBI and accelerated, long-term Aβ accumulation, independent of gender, which may be triggered by acute axonal injury and a buildup of Aβ oligomers. However, when the BII increases, allowing the brain to metabolically recover between injuries, accelerated Aβ accumulation does not occur.

CONCLUSIONS

The impetus for this dissertation was the growing concern over the long-term effects of repeat concussions. Most of the focus has centered on professional athletes, but these professionals often experience their first concussion during adolescence. Furthermore, the incidence of mild traumatic brain injury (mTBI) during this critical developmental period is exceedingly high, with 70.5% of mTBI occurring in adolescents between 10-19 years old (CDC MMWR, 2011). MTBI is a public health concern in its own right. However, when the potential relationship between RTBI and neurodegenerative disease was first described in 1928 (Martland) and re-emerged in the 1960's with *dementia pugilistica*, or "Punch Drunk" in boxers (Miller, 1966), the problem grew exponentially. To date, several studies have probed the potential link between TBI and other neurodegenerative diseases such as Parkinson's disease (Goldman et al., 2006), Amyotrophic Lateral Sclerosis (Chen et al., 2007), and recently, Chronic Traumatic Encephalopathy (McKee et al., 2009). These neurodegenerative diseases are often considered distinct pathological conditions, however, there are several overlapping

mechanisms between them such as the accumulation of toxic proteins, inflammation, and metabolic disturbances, so insight in to one disease will likely pave inroads in to the others. AD affects 4.7 million people in the U.S. (Hebert et al., 2013) and this figure is expected to rise considerably in the next 25 years. Those statistics, combined with the established risk association between moderate/severe TBI and AD, justifies the necessity for research in the area of repeat mild TBI and AD.

The current body of TBI-AD literature has several limitations, making an understanding of the relationship somewhat elusive. First, the epidemiological studies linking TBI and AD are based on self-reports of concussion history. Recall bias may affect the number of injuries reported. This factor is further confounded by the fact that many concussions are undiagnosed or go unreported. Second, the experimental research focuses on single mTBI, or in the rare instance, RTBI, in the adult brain. Adolescent studies are nonexistent. Overall, there is a need for prospective designs at both the clinical and pre-clinical levels.

This dissertation established a link between RTBI in the adolescent brain and accelerated Aβ pathogenesis and provided potential mechanistic explanations for this relationship. It also highlighted the long-term importance of increasing the brain impact interval (BII), or the period of time between repeat concussions. Four mild traumatic brain injuries during adolescence with a BII of 24 hours were correlated with accelerated Aβ burden in the hippocampus approximately 10.5 months later. However, when the BII increased to 72 hours, allowing the brain to metabolically recover between injuries, Aβ

burden was not statistically different from shams. This remarkable finding clearly demonstrates the long-term, cumulative effects of RTBI in close time proximity, but highlights the benefit of maximizing the recovery time between repeat injuries.

The major challenge in the AD literature, and as a result of the aforementioned findings, is to unmask the mechanisms responsible for the RTBI-AD link. One possible explanation was proposed by Chen and colleagues (2004), who posited that RTBI causes traumatic axonal injury (TAI), leading to axonal transport disruption and an accumulation of APP cleavage enzymes (BACE1 and PS1). This results in an increase of Aβ oligomers, which leave the axons and ultimately aggregate as plaques. Based on this theory, it was critical to examine the presence of TAI and accumulated BACE1, PS1, and Aβ oligomers. Diffusion tensor imaging revealed decreased anisotropy subacutely after $4RTBI_{24}$ that resolved by 6 months after injury. This TAI was not seen in the single or double injured groups, suggesting a detrimental cumulative effect. Biochemical analysis of the intra-axonal proteins determined that BACE1 did not change acutely after injury and PS1 decreased. However, the APP cleavage products, Aβ oligomers, were elevated after 4RTBI. This increase in Aβ oligomers, despite the lack of an increase in the substrate required to form Aβ, suggests a potential impairment in the Aβ clearance mechanism, a point of future study.

AD and TBI are two intricately complex disease states, but this research adds to the growing understanding of how they may be linked. Based on the literature and the collective data from this work, I am proposing a comprehensive map of interactive

mechanisms that might explain the accelerated Aβ pathology chronically after RTBI (Figure 28). Importantly, this accelerated Aβ pathology was only observed with a short, 24-hour BII as opposed to a longer, 72-hour BII. The proposed central mechanistic difference between these two groups is glucose metabolism, with previous studies showing CMRglc depression greater in magnitude and duration after repeat injuries spaced 24 hours apart (Prins et al., 2013). AD does not develop linearly with a single event triggering a molecular change leading to debilitating pathology and behavioral deficits. Instead, it is likely caused by a web of interrelated cellular and molecular processes triggered by environmental and genetic factors. The relationship between these processes is based on the large body of AD literature and is represented by blue arrows in Figure 28. Briefly, the central protein, Aβ, increases as a result of the prolific effect of inflammation on APP. However, Aβ also exacerbates inflammation (Jung et al., 2015) and blood brain barrier weaknesses (Glushakova, 2014), creating a feedforward loop. Then, Aβ causes extracellular glutamate release, which overstimulates the NMDA receptor, resulting in an upregulation of intracellular Ca^{2+}, redox events, oxidative stress, and mitochondrial dysfunction (Benilova et al., 2012). This impairment causes metabolic depression, which then leads to dysfunctional synapses, decreased long-term potentiation (LTP), and cognitive decline.

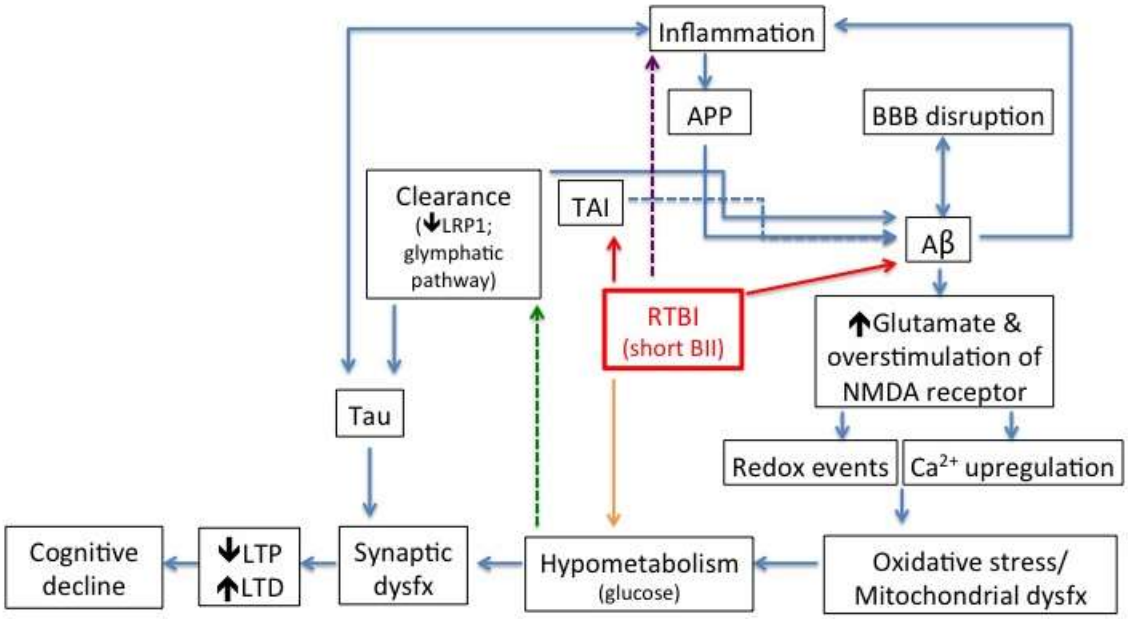

Figure 28. Schematic illustrating the proposed mechanistic relationship between $4RTBI_{24}$ and accelerated AD pathology. Blue arrows represent a simplification of the early processes involved in AD, based on the collective literature. Red solid arrows represent the findings of this study; specifically, $RTBI_{24}$ resulted in traumatic axonal injury (TAI) and increased soluble Aβ and Aβ plaques. Our previous work showed that RTBI exacerbated hypometabolism (orange solid arrow). The three main proposed mechanisms for these relationships involve 1) hypometabolism, 2) impaired clearance (green dotted arrow) and 3) inflammation (purple dotted arrow). These perturbed processes may hasten the AD neurodegenerative process. This schematic lays the framework for future studies.

Based on this work, I propose that early RTBI perturbs the intricate web of AD-related pathways at various points, potentially hastening the disease process. For example, this dissertation demonstrates the correlation between $RTBI_{24}$ and both soluble and insoluble Aβ, as well as traumatic axonal injury (see red arrows in Figure 28). Furthermore, our previous work showed the effect of RTBI on hypometabolism (Prins et al., 2013). Other studies have demonstrated a correlation between mTBI and blood brain barrier disruption (Marchi et al., 2013) cerebral metabolism (Prins et al., 2013), tau (Johnson et al., 2012; Tran et al., 2011), glutamate release (Katayama et al., 1990), oxidative stress and mitochondrial dysfunction (Vagnozzi et al., 2007). Interestingly,

TBI has also been shown to impair cerebral clearance mechanisms, such as the recently discovered glymphatic pathway, which leads to an increase in tau and synaptic dysfunction (Iliff et al., 2014). Of particular importance is the finding that $4RTBI_{72}$ (repeat injuries with a longer BII) increases acute Aβ oligomers, but not long-term Aβ plaques. Perhaps the likely short-lived hypometabolism with a BII of 72 hours, as opposed to the longer-lasting hypometabolism with a BII of 24 hours, prevents the acceleration of AD-related pathways. Overall, there are many points during the AD neurodegenerative process where RTBI may exert its influence and accelerate the disease. However, BII may determine the extent of the RTBI-AD association. Future research should examine the effect of BII on the various processes presented in Figure 28.

Concussion research is often misinterpreted as being anti-sport, or at least anti-football, which is not an accurate assessment. There is tremendous physical, mental, and social value to be gained from participating in sport, especially during adolescence. The critical issue is how to better prevent, diagnose, and treat repeat concussions. While the rules of the game can ameliorate chances for injury, injury management is critical as well. One necessary component of injury management is the issue of return-to-play (RTP), or when an athlete can resume playing his or her sport after a concussion. RTP guidelines based on empirical evidence of the BII might mitigate the acute and chronic effects of repeat concussions, which were demonstrated in these studies.

One factor confounding the adherence to RTP guidelines is the culture of sport. We have come a long way since 1983 when 69% of athletes who experienced a loss of consciousness returned to play in the same game (Gerberich 1983). However, there still exists a culture of "toughness" that resists the sense of vulnerability that is associated with injury, especially "invisible" brain injury. Many athletes fail to report their symptoms (Dziemianowicz et al., 2012) because of the desire to continue playing (Chrisman et al., 2013). In fact, one study demonstrated that only 47.3% of high school football players reported their concussion-like symptoms because they did not understand what a concussion was, did not appreciate the severity of concussions, or did not want to be sidelined (McCrea et al., 2004). Clearly, efforts to educate athletes on the effects of concussion, based on scientific evidence, need to be continued.

Rule changes in sport can also carry high potential for preventing repeat concussions. In a publication of effective concussion risk-reduction strategies in sport, Benson and colleagues (2013) demonstrated that rules changes were the best strategy (among improved helmets/mouth guards and legislation) for reducing concussion risk. Fair-play rules reduced concussions in high school hockey players (Roberts et al., 1996) and the elimination of body checking decreased concussion rates in pre-adolescent hockey players (Macpherson et al., 2006; Emery et al., 2010). Similar improvements have been anecdotally reported after the implementation of rules limiting helmet-to-helmet contact in football. Although technology continues to advance, leading to new helmet or mouth guard designs, as well as real-time accelerometers, the effect of these

tools on either diagnosing concussion or mitigating its consequences are largely unproven (Benson et al., 2013; Bonfield et al., 2015).

Concussion prevention, diagnosis, and management, especially among the highly at-risk adolescent population, will continue to require a multidimensional approach. Changes in the culture and rules of sport as well as continued concussion education and RTP guidelines can help prevent these mild traumatic brain injuries and ultimately decrease the risk of accelerated neurodegenerative disease. Most importantly, basic science research, such as the experiments performed in this dissertation, can provide invaluable insights in to the mechanisms involved with RTBI and neurodegeneration. Animal studies have an advantage over clinical studies in that the timing of BII can be tightly controlled and the effect of RTBI in adolescence on neurodegeneration in late-adulthood can be completed within a shorter timeframe. However, all concussion research must be carefully controlled, replicated, properly interpreted, and translated to the non-scientific community of athletes, parents, coaches and athletic trainers in a responsible and productive manner.

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