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The Effect of Grapes in Mice with Alzheimer's Disease and Brain Injury

Nikita Mistry

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The Effect of Grapes in Mice with Alzheimer’s Disease and Brain Injury

by

Nikita Mistry

A Thesis submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in Experimental Psychology

December 2014
Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Doctor of Philosophy.

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Richard E. Hartman, Associate Professor of Psychology

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Paul Haerich, Professor of Psychology

_________________________
Viorela Pop, Clinical Research Coordinator, University of California, Irvine
ACKNOWLEDGEMENTS

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## ABBREVIATIONS

<table>
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<tr>
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<th>Full Form</th>
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<tr>
<td>Aβ</td>
<td>Amyloid Beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid Precursor Protein Intracellular Domain</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-Cell CLL/Lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral Amyloid Angiopathy</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled Cortical Impact</td>
</tr>
<tr>
<td>CTFα</td>
<td>C-terminal Fragment Alpha</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double Distilled water</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s Disease</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GSE</td>
<td>Grape Seed Extract</td>
</tr>
<tr>
<td>HJ3.4</td>
<td>Monoclonal HJ3.4 Amyloid Beta Antibody</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary Tangle</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative Stress</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End Products</td>
</tr>
<tr>
<td>sAPPα</td>
<td>N-terminal Amyloid Precursor Protein Alpha</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
</tr>
<tr>
<td>Thio-S</td>
<td>Thioflavin S</td>
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ABSTRACT OF THE THESIS

The Effect of Grapes in Mice with Alzheimer’s Disease and Brain Injury

by

Nikita Mistry

Doctor of Philosophy, Graduate Program in Experimental Psychology
Loma Linda University, December 2014
Richard E. Hartman, Chairperson

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder resulting in cognitive and memory deficits and deposits of amyloid beta (Aβ) in the cortex and hippocampus are thought to be significant contributors to the disease process. Increasing evidence suggests that foods rich in polyphenols, such as pomegranates and grapes, may have neuroprotective effects in both rodents and humans. Our previous research has shown that a pomegranate-enriched diet decreased Aβ plaque load and improved behavior in Tg2576 mice. In this study, we determined whether a grape-enriched diet altered Aβ neuropathology and TBI in PSAPP mice. Mice received either craniotomy or a moderate TBI. Naïve mice (non-injured) underwent anesthesia. Mice consumed either a grape-enriched or a control diet for 8 weeks. Behavioral tests were administered 2 weeks after injury. CCI mice showed significant motor and memory deficits, with no effect of diet. The grape-enriched diet trended towards the reduction of lesion volume and motor deficits in craniotomy mice. Most surprising, CCI reduced fibrillar Aβ plaque load while diet did not have an affect on AD neuropathology. These results suggest that brain injury may be neuroprotective against the progression of AD.
CHAPTER ONE
INTRODUCTION

Alzheimer’s disease (AD) is the most common neurodegenerative disease and can cause brain atrophy and behavioral deficits. AD neuropathology usually begins in the temporal lobes, leading to the first signs of memory and emotional problems and eventually progresses to the frontal and parietal lobes, further contributing to personality changes, language difficulties, impaired decision-making and motor impairment.

Neuropathology consists of the aggregation of misfolded proteins in the brain’s extracellular space (plaques of amyloid beta [Aβ]) and within neuronal axons and dendrites (neurofibrillary tangles [NFTs] of tau protein). The Aβ plaques disrupt neuronal signaling (Uryu et al., 2002), causing the breakdown of necessary communication of networks in the brain. Aβ deposition generally begins in the temporal lobe within and around the entorhinal cortex and the hippocampal formation, which impacts learning and memory, then spreads to the cortical and subcortical areas that impact language, executive, and motor functions. The phosphorylation (addition of a phosphate group) of the tau protein regulates its activity to bind to microtubules and stimulates the assembly and the maintenance of the axonal structure. In AD, tau is hyperphosphorylated (too many phosphate groups attach) and this causes tau to lose its normal biological activity. Tau proteins aggregate and form tangles (NFTs) within the axon, eventually collapsing the axonal structure and transport mechanisms (Wakasaya et al, 2011). Tau proteins aggregate and form tangles (NFTs) within the axon. Both Aβ and NFTs are associated with increased oxidative stress, inflammation, glial activation, synaptic loss, apoptosis (cellular suicide), mitochondrial dysfunction, and vascular plaque deposition in the brain.
Several risk factors for the disease include aging, genetic mutations, diet, and brain injury. Genes associated with early-onset / familial AD (FAD) contributes to only 10% of AD cases. FAD symptoms typically develop during the 50s, and sometimes as early as 30 years of age. Behavioral and neurological assessments are commonly used to diagnose AD, however, other neurological diseases may present similar symptoms. Unfortunately, typical behavioral symptoms are not observed until significant levels of neurodegeneration have occurred. Because this process may take decades, a concrete diagnosis of AD can only be confirmed after post-mortem neuropathological analysis.

**Amyloid Precursor Protein Gene Family**

Mutations of presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*), and/or *APP* can enhance cleavage patterns in support of Aβ (Karran, Mercken, & De Strooper, 2011). The human *APP* gene is located on chromosome 21 and is expressed in many tissues and organs, including the brain and spinal cord. It encodes amyloid precursor protein (APP), which is a protein partially embedded in the cell membrane with a large extracellular domain, and mutations in *APP* are thought to play a significant role AD pathology. More than 25 *APP* mutations have been identified that cause FAD and the deposition of Aβ in the brain’s blood vessels (cerebral amyloid angiopathy; CAA). These mutations alter the genetic code and will ultimately lead to the production of abnormal APP that has affinity for aggregation. Approximately 10 percent of these mutations are responsible for FAD. In CAA, blood flow becomes more constricted due to the buildup of amyloid proteins inside vasculature, leading to cerebral microbleeds (Carrano et al., 2011). This increases the risk of stroke, oxidative damage and blood brain barrier dysfunction drastically. Because this mutation is found in approximately ¼ of adults with AD, CAA mouse models (e.g., the
SAMP8 mouse) have been developed to better define the similarities between the diseases. Briefly, this model mimics the altered phospholipid content in cell membranes and early abnormalities in learning and memory that are characteristic in AD (Morley, 2003).

Presenilins are a family of transmembrane proteins that function in the processing and trafficking of membrane-bound proteins in neurons. Mutations in PSEN1 and PSEN2 have been identified in the majority of early-onset autosomal AD. Presenilins 1 & 2 is thought to regulate the cleavage activities of many proteins in the cell, including gamma secretase (Hutton & Hardy, 1997; Sherrington et al., 1995). These mutations disrupt normal APP processing, leading to the overproduction of Aβ (Sherrington et al., 1996). Some research suggests that blocking γ-secretase may decrease Aβ induced oxidative stress by increasing mitochondrial function (Sheng et al., 2009; Kurz et al., 2010) and promote normal cellular activity and viability. However, these drugs have adverse side effects. Blocking gamma secretase also leads to the inhibition of other crucial cellular processes and may cause abnormalities and cytotoxicity in the gastrointestinal tract, thymus, and spleen in rodents (Imbimbo, 2008).

Despite the large number of published studies on APP, there is still no clear consensus on the protein’s function. A recent review by Dawkins and Small (2014) summarizes the major ideas relating to the function of the protein, suggesting that normal APP interacts both intracellularly and extracellularly to regulate various signal transduction mechanisms. More specifically, the physiological role of APP may involve neural stem cell development, neuronal survival, neurite outgrowth, and neurorepair. Evidence also suggests that APP may play an important role in synapse formation, neural
plasticity, and axonal transport. APP accumulates in presynaptic terminals and growth cones and may function to inhibit synapse formation. In mouse models where APP is genetically removed (APP-/-), a number of deficits are associated with altered synaptic function in hippocampal neurons, such as hypersensitivity to kainate-induced seizures, alterations in dendritic spine density, and reduced performance in tests of spatial memory (Steinbach et al. 1998; Dawson et al. 1999).

APP is delivered to the surface of cell membranes where it is metabolized into a number of protein fragments by the alpha- (α), beta- (β), and gamma- (γ) secretases, yielding a variety of amino- (N-) terminal (free floating) and membrane anchored carboxy- (C-) protein fragments. APP that fails to be cleaved by α-secretase can be internalized into endocytotic compartments (engulfed) and subsequently cleaved to generate Aβ (Kamenetz et al., 2003). In general, APP cleavage tends to follow one of two pathways: the amyloidogenic pathway (producing the Aβ peptide) and the non-amyloidogenic pathway (preventing production of the Aβ peptide; Karran, Mercken, & De Strooper, 2011).

**Amyloid Beta Production**

Amyloid beta peptides (39-43 amino acids each) are formed from APP by specific enzymatic cleavage sequences into smaller protein fragments (peptides). In a healthy brain, these protein fragments are broken down and eliminated. In an AD brain, Aβ fragments gradually accumulate and trigger downstream events causing functional neuronal deficits, oxidative stress (OS), and cell death. The amyloid cascade hypothesis (Figure 1) suggests that mutations enhance the formation of soluble toxic Aβ oligomers
and/or deposited fibrillar amyloid peptides. With the development of Aβ aggregates, OS levels in the brain are amplified, leading to cellular degeneration, followed by increased production of APP and its subsequent cleavage via the amyloidogenic pathway. As a result, the cycle continues to generate Aβ, which aggregates into plaques, causing the development of NFTs, ultimately leading to severe neuronal degeneration.

Figure 1. The amyloid cascade hypothesis. Genetic and spontaneous mutations of APP lead to Aβ aggregation and stress, eventually leading to NFTs and further degeneration.

The Amyloidogenic Pathway

APP is first cleaved by β-secretase near the membrane surface followed by γ-secretase to yield Aβ monomers. The initial cleavage of APP by β-secretase yields a soluble N-terminal (sAPPβ) fragment and membrane bound C-terminal end (CTFβ). Next, the cleavage of the membrane bound CTFβ fragment by γ-secretase yields two
more fragments: Aβ and CTFγ, a membrane bound APP intracellular domain (AICD). The specific cleavage site of γ-secretase may result in different peptide sequences ranging from 39 to 43 amino acids. Amyloid beta sequences of 40 (Aβ40) and 42 (Aβ42) are commonly found in AD, with Aβ42 more likely to aggregate into β-pleated sheets, compared to shorter soluble isoforms. Aβ40 remains in a less toxic monomeric peptide sequence. With large enough concentrations of these monomers begin to aggregate to form large diffuse Aβ fragments called oligomers. Strands of Aβ42 also tend to cluster together and form oligomeric aggregates that become tangled with neighboring cells and synapses. As more oligomers cluster together, the structure undergoes conformational changes, leading to the formation of amyloid β-sheets (Askarova, Yang & Lee, 2011). The stacking of these sheets are called fibrillar amyloid β-sheets, are extremely dense and resistant to degradation (Askarova, Yang & Lee, 2011). The deposition of these fibrillar, more end stage AD plaques are associated with oxidative stress, disrupting neuronal signaling, and contributing to neuritic degeneration (Han et al., 2006; Jacobsen et al., 2006).

**The Non-Amyloidogenic Pathway**

APP is cleaved by α-secretase followed by γ-secretase to yield a non-toxic protein. Cleavage by α-secretase yields a soluble N-terminal (sAPPα) fragment and a membrane bound C-terminal end (CTFα). This results in larger sAPPα chains that reduce the chances of the formation of Aβ monomer fragments. Also, sAPPα may be involved in the formation of synapses, neurite outgrowth and neuronal survival, and are considered to be neuroprotective (Chasseigneaux & Allinquant, 2012). Finally, γ-secretase cleaves
CTFα to yield a soluble p3 fragment and a membrane bound AICD fragment (Askarova, Yang & Lee, 2011). Because the full-length Aβ protein is not produced, these cleavage sites release fragments that may contribute to neuronal survival.

**Oxidative Stress**

Oxidative stress (OS) occurs from the imbalance in the normal reactive state of tissues that can cause toxic effects through the production of peroxides and free oxygen radicals, which in turn damage cellular components including proteins, lipids and deoxyribonucleic acid (DNA). Although, OS increases with age, brain injuries and/or neurological diseases exacerbate its concentration and causes more harm in the brain. Research in AD has recently demonstrated compelling evidence on the importance of oxidative processes and pathology. Several studies have identified the roles oxidative stress, free radical damage, and inflammation has in pathology and possibility the etiology of AD. Oxidative species and free radicals may initially be products of mitochondrial dysfunction and imbalances of trace elements such as iron and copper (Perry, Cash, & Smith, 2002). These metals generate reactive oxygen species that ultimately disrupt metabolic cellular processes (Honda, Casadesus, Peterson, Perry, & Smith, 2004). Increased oxidative species may trigger cytoskeletal modifications in susceptible neurons (leading to NFTs) (Honda et al., 2004; Perry et al., 2002) and the up-regulation of presenilin 1 to produce Aβ (Oda, Tamaoka & Araki, 2010), consequently leading to the development of AD. Aβ induced toxicity continues to disrupt of neural networks and affects long-term potentiation and memory consolidation, eventually leading to cognitive deficits (Jacobsen et al., 2006).
Brain injury induces inflammation and toxicity with increased free radical production in the brain. With each additional injury, the cytotoxic environment continues to rise, eventually leading to neurodegenerative diseases. For example, traumatic brain injury accelerates the formation of Aβ deposits (Uryu et al., 2002), damages hippocampal neurons, and induces learning and memory behavioral deficits (Han & Chung, 2006). This is especially evident in athletes such as boxers and football players that will present cognitive symptoms and plaque pathology similar to AD and other dementias (Mitsis et al., 2014; Jordan, 2000). These types of repetitive concussive injuries can further accelerate OS levels and chronic inflammatory responses, leading to continued tissue degeneration.

**Mouse Models in Alzheimer’s Disease**

Many neurological diseases are difficult to study using human models because of the significant risks, time, and ethical issues involved in experimental studies. As a result, many researchers use animal models to investigate disease pathologies, therapies, and cures. Transgenic mice with Alzheimer’s-like pathology have been developed in an attempt to understand the disease in its entirety at a faster pace. These mice develop the pathological (Hsiao et al., 1996; Jacobsen et al., 2006; McGowen et al., 1999) and behavioral hallmarks (Liu, et al., 2008) of AD similar to those seen in adults. Commonly used mouse models are described below.

The first transgenic mouse created was the PDAPP model. These mice express a double APP mutation associated with autosomal dominant forms of human AD. Plaque deposition occurs at approximately at 6 months in the cortex, followed by progression to
the hippocampus and corpus callosum. These mice have a 5 to 10 fold increase in Aβ production compared to controls and noticeable learning and memory impairments before the plaque deposition. Behavioral deficits and hippocampal volume reductions characteristic in this model are similar to those found in human AD patients (Games et al., 1995).

The Tg2576 (APP$_{695}$SWE) mice model is used to study the early-onset of AD. These mice express a human APP from a Swedish family, which results in a double mutation in APP$_{695}$ (Lysine$^{670} \rightarrow$ Asparagine, Methionine$^{671} \rightarrow$ Leucine). These mice have 5 times more soluble Aβ and 14 times the toxic Aβ plaques compared to humans (Hsiao et al., 1996). Plaque deposition begins in the entorhinal and piriform cortices, thereafter proceeding to the cingulate and motor cortices (McGowen et al., 1999), with learning and memory impairments are observed by 9 months (Hsiao et al., 1996).

A multi-gene transgenic mouse model PSAPP is also used to study the early-onset of AD. This model involves the crossing of the human PSEN1 mutation with Tg2576 mice. Behavioral deficits are detectable beginning around 6 to 7 months. Differing from Tg2576 mice, plaque deposition in these mice begins around 5 months in the cingulate and motor cortices, thereafter proceeding to the entorhinal and piriform cortices (McGowen et al., 1999).

A triple transgenic mouse model (3xTg-AD) has been newly developed study AD. This model aims to mimic the neuropathology of the disease in humans very closely. Mice harbor 3 mutant transgenes: human PS1$_{M146V}$, double mutation APP$_{SWE}$, and human tau$_{P301L}$. Mice develop progressive and age-dependent Aβ and tau pathology, with Aβ deposition around 6 months followed by tau aggregates at 12 months. The Aβ deposits
being in the cortex, then spreads to the hippocampus with age. In comparison, tau pathology begins in the hippocampus and progresses to the cortex (Oddo, Caccamo, Kitazawa, Tseng & LaFerla, 2003).

**Risk Factors in Alzheimer’s Disease**

**Genetics**

Autosomal mutations in genes that encode APP and presenilins predispose individuals to develop early-onset FAD by either by increased levels of APP in the brain or accelerating processing of APP to form Aβ. A major genetic risk factor for the more common late-onset AD is carrying a copy of the $\varepsilon 4$ allele of the gene that encodes apolipoprotein E ($APOE$). APOE is a low-density lipoprotein that typically functions in the movement of lipoproteins, fat-soluble molecules, and cholesterol within the bloodstream and seems to play an important role in AD pathogenesis. APOE is primarily produced by astrocytes, microglia, and may interact a number of receptors as well as structures in the brain.

There are multiple isoforms of the $APOE$ gene: $\varepsilon 2$, $\varepsilon 3$, AND $\varepsilon 4$. Those with the $APOE4$ gene are more likely to develop AD and two copies of the gene may accelerate Aβ processing and increase plaque burden and toxicity (Askarova, Yang & Lee, 2011), thus presenting a higher risk for developing the disease. Carriers of the $\varepsilon 4$ allele have early signs of degeneration in the brain such as reduced cortical thickness in the entorhinal cortex and subiculum (Burggren et al., 2008), age related reduction in hippocampal volume (Jak, Houston, Nagel, Corey-Bloom, & Bondi, 2007; Lind et al., 2006), and impaired performance on cognitive learning and memory tasks (Lind et al.,
Research has shown that mice deficient in APOE (−/−) do not express astrocytes around the blood brain barrier, leaving the brain more vulnerable to injury and subsequent damages (Methia et al., 2001). This causes transport mechanisms to be lost and/or allows other endogenous substances to enter the brain. As such, APOE plays a critical role in the maintenance of brain health.

Copies of ε2 and ε3 alleles are known to be protective against AD. The ε3 allele is a neutral and asymptomatic to any known diseases. Although the ε2 allele does not play a role in AD, it is involved in lipid and cholesterol transport and dysfunction that may increase the risk for cardiovascular diseases (Civeira et al., 1996).

**Diet**

Evidence suggests that diets can have serious implications in altering the physiological functions of the brain and increasing the risk of psychiatric and neurological disorders. For example, high fat diets promote vascular atherosclerosis (Franciosi et al., 2009; Lemieux et al, 2010) and behavioral deficits (Li, Cao, Garber, Kim & Fukuchi, 2003; Liu et al., 2008) by altered cholesterol and phospholipid levels in cell membranes. These changes can modulate activity of membrane bound enzymes including β- and γ-secretases that enhance Aβ production in the brain significantly. High-cholesterol diets have been shown to increase Aβ pathology in the brains of rabbits (Lemieux et al, 2010; Ronald et al., 2009; Wu et al., 2003) and APP transgenic mice (Hooijmans et al., 2007; Pedrini et al., 2009; Shie, Jin, Cook, Leverenz & LeBoeuf, 2002). Specifically, high intake up-regulates APOE, which in turn up-regulates Aβ plaque deposition in rabbits (Wu et al., 2003). High fat diets also increase tau pathology.
(Glöckner, Meske, Lütjohann & Ohm, 2011) and astrogliosis (Crisby, Rahman, Winblad & Schultzberg, 2004) in APOE (−/−) deficient mice.

**Traumatic Brain Injury**

Traumatic brain injuries (TBI), such as those commonly caused from falls, contact sports, vehicular accidents, violence, and military combat may lead to long-lasting cognitive and motor deficits, and increase the risk of developing future neurological disorders. An estimated 1.7 million people are affected by TBI annually and is the leading cause of death and disability in both combat and civilian circumstances (Yu, Watt, & Mohan, 2013). TBI affects all ages and socioeconomic classes and may result in short-term and/or long-term motor, cognitive, and emotional deficits (Budinich, Tucker, Lowe, Rosenberger, & McCabe, 2013). Despite the high incidence of TBI, few preventative and treatment options are available (Chauhan, Gatto, & Chauhan, 2010).

Sources of TBI include any external mechanical force, penetration by an object, and direct or indirect impacts. TBI may be classified based on severity (e.g. mild, moderate, or severe), injury location, time of lost consciousness, with symptoms lasting from seconds to possibly years. Recoveries of motor and cognitive deficits are dependent on the severity of the injury (Huh et al., 2011). Physiological responses to brain injury include increased OS, excitotoxicity, mitochondrial dysfunction, inflammation, cellular dystrophy, and other processes that will ultimately result in neuronal death. The initial impact results in the displacement of the structures in the brain. This includes contusion, damage to the blood vessels, axonal shearing, and compromised blood brain barrier (BBB) and meninges. Secondary injuries occur in the hours and days following the
primary injury, where the brain’s activated metabolic networks remain heightened and continue to inflict damage over time. Secondary injuries include but are not limited to insufficient blood and oxygen flow to the brain and cranial swelling often cause the persistent long-term deficits.

Mild to moderate TBI leads to impairments in executive processing, long-term memory consolidation (Miotto et al., 2010), strategy and decision-making (Miotto et al., 2010; Noordt & Good, 2011), episodic memory, and in verbal recognition and verbal episodic memory tasks (Miotto et al., 2010). Severe TBI leads to cognitive and motor deficits that are more damaging and long lasting, resulting from widespread tissue degeneration. These occur as a result from sustained cerebral contusions, diffuse axonal injury, ischemic brain damage, symmetrical ventricular enlargement, and an increase in intracranial pressure (Adams et al., 2011).

Experimental models of TBI have been developed to mimic physiological and behavioral deficits found in humans. Controlled cortical impact (CCI) is commonly used as a rodent model of TBI that allows independent control of many mechanical parameters and produces many of the features found in TBI (Dixon, 2009). A piston is used to directly impact the cortex and will result in a variety of acute and long-term behavioral impairments. This model is essential in delivering a focal, more specific TBI due to the fact that severity can be manipulated by varying the piston’s speed and/or depth, with larger depth and speed inducing larger locomotor and cognitive deficits.

Diffuse axonal injures are more widespread and occur regardless of severity (Adams et al., 2011). These are characterized by extensive lesions in the white matter tracts that disrupt nerve impulses throughout the brain, resulting in cognitive impairment
Unfortunately, the risk of experiencing more tissue damage and behavioral deficits increases with each repeated TBI. Individuals who have experienced one brain injury are three times as more likely to experience a second brain injury (DeFord SM, Wilson MS, Rice AC, Clausen T, Rice LK, Barabnova A, et al). A recent study assessed the temporal development of neuropathology in a CCI rat model. This study used MRI techniques to investigate the effects of repeated mild TBI (1 impact to each cortical hemisphere (Donovan 2012)). Tissue damage was exacerbated following the second mild TBI, most notably when injuries were seven days apart (Donovan 2012). The repetitive nature of these injuries accelerated neuronal damage, oxidative stress, (Han et al., 2006) and cognitive impairments (Uryu, et al., 2002). Strikingly, these cortical white matter abnormalities are observed as late as 60 days post injury (Donovan 2014). These studies suggest that the brain remains vulnerable to a subsequent injury for a period of time, increasing the probability of additional tissue damage and behavioral deficits (Yu S, Kaneko Y, Bae E, Stahl CE, Wang Y, van Loveren H, et al).

TBI produces behavioral deficits (Creed, DiLeonardi, Fox, Tessler, & Raghupathi, 2011) and cellular pathology (Alder, Fujioka, Lifshitz, Corckett, & Thakker-Varia, 2011; Huh, Widing, & Raghupathi, 2011; Li, Li, Feng, & Gu, 2011) similar to adults with AD. Athletes such as football players and boxers are at an increased risk for developing early onset AD with each repetitive TBI. Interestingly, individuals with TBI have shown evidence of extensive cortical Aβ plaque deposition (Roberts et al., 1994). Aβ plaques also form immediately post injury in the cortex (Gentleman et al., 1997; Valle et al., 2011) and continue to form several weeks after (Uryu et al., 2002) as a result of the heightened expression of APP (Tatsuki et al., 2009). Increased concentrations of soluble
Aβ42 in the cortex (DeKosky et al., 2007) and cerebral spinal fluid (Olsson et al., 2004) have also been recorded. It is this gradual accumulation of Aβ that causes pathophysiological changes in the brain that generally increases the risk of developing AD neuropathology. Unfortunately, few pharmacological treatments are available for TBI and those that are available have significant adverse side effects. These treatments are typically implemented after the injury has occurred and may be too late in preventing further neurodegeneration. Consequently, TBI may predispose individuals to develop neurodegenerative diseases such as Alzheimer’s disease, earlier than the normal aging population.

**The Blood Brain Barrier**

The neurovascular unit is a complex network of neurons, vascular cells (endothelia, pericytes, smooth muscle cells), and glia (astrocytes, microglia, and oligodendroglia). The vascular cells and glia form the anatomical, biochemical, and barrier systems of the central nervous system. The blood brain barrier (BBB) is composed of endothelial cells that are surrounded by basement membranes, pericytes, and astrocytic endfeet. These characteristics in addition to tight junctions support the integrity of the BBB. There is growing evidence that brain injury, whether its ischemic, hemorrhagic, or traumatic leads to dysfunction of BBB. Changes in the permeability of the BBB are observed after injury and are thought to contribute to the degeneration of tissue in the brain. For example, failed clearance mechanisms of the BBB lead to Aβ plaque deposition in the brain, opening the route to further neuronal degradation.
Receptor for Advanced Glycation End Products

The human receptor for advanced endproducts (RAGE) is a multiligand cell surface protein that is involved in inflammatory and immune responses. Rage is expressed by a number of cell types including neurons, microglia, astrocytes, and endothelial cells in the central nervous system. RAGE is a receptor for multiple proteins including Aβ. RAGE that is expressed in the surface capillaries of endothelial cells in the BBB regulate the influx of peripheral Aβ into the brain (Mackic et al., 1998). The inflammatory response triggered from AD (Lue et al., 2001), leads to higher OS levels and increased Aβ transport across the BBB (Deane et al., 2006). Transportation further signals the up-regulation of RAGE dramatically, leading to higher concentrations of the receptor and Aβ in the hippocampus (Donahue et al., 2006) and Aβ in blood capillaries (Jaynes & Provias, 2008), ultimately leading to neuronal loss. Since Aβ mediation by RAGE contributes to neuronal dysfunction, blocking the receptor may ameliorate cellular toxicity and reduce Aβ levels (Carrano et al., 2011), thus slowing the progression of AD.

Astrocytic Activation

Astrocytes are star-shaped glial cells located in the brain and spinal cord that are critical to the maintenance and functioning of the brain. These cells envelope synapses between neurons, surround endothelial cells in the BBB, play a role in repair and scarring from injury, and control the extracellular space and its contents in the brain. Astrocytes become activated (reactive) in response to inflammation or to CNS pathologies, such as stroke, traumatic brain injury, and AD. Reactive astrogliosis occurs when the astrocytes surround a lesioned area of interest and undergo morphological changes that include
hypertrophy, branching of processes, and proliferation (Perez, et al., 2010; Rodríguez, Olabarria, Chvatal, & Verkhratsky, 2009) in an attempt to isolate the area and prevent further cellular degradation. Thereafter, astrocytes replace the damaged tissue with a glial scar (Rodríguez et al., 2009) to contain the damaged area.

In neurodegenerative diseases such as AD, astrogliosis begins several months prior to Aβ plaque deposition in APP transgenic mice (Heneka et al., 2005; Mei, Ezan, Giaume, & Koulakoff, 2010) and continues to increase as the disease advances. Activated astrocytes surround and become intertwined with Aβ plaques (Rodríguez et al, 2009) and attempt to separate them from nearby neurons (Wegiel et al., 2000). In a study where non-transgenic rats were injected with Aβ1-42 oligomers, significant activated astrocytes were found around the injection site compared to control Aβ42-1 animals (reversed peptide without fibrillogenic properties) (Perez et al., 2010). Astrocytes also surround and ingest more diffuse oligomeric Aβ than fibrillar Aβ (Nielsen et al., 2010), suggesting its role to minimize damage triggered from AD.

**Polyphenolic Compounds**

Many studies suggest that diets high in antioxidants and polyphenols attenuate neuronal degradation. Polyphenols are water soluble, organic chemicals that are high in antioxidant and anti-inflammatory properties. Commonly found in a wide range of fruit and spices, the supplementation of these compounds into diets may be an advantageous method to slowing neurological diseases, compared to moderately effective pharmaceutical medications.
Epidemiological studies suggest the role of polyphenolic compounds in attenuating age-related deficits, with recent attention directed towards a well balanced, high polyphenol diet. The Mediterranean diet is simplistic and typically abundant in fresh fruits, vegetables, breads, beans, nuts, seeds, olive oil, cheese, yogurt, seafood, and wine. High adherence to the Mediterranean diet is associated with lower cognitive decline (Féart, Samieri, & Bargerger-Gateau, 2010; Féart, Pérès, Samieri, Letenneur, Dartigues & Bargerger-Gateau, 2011; Scarmeas, Stern, Mayeux, Manly, Schupf & Luchsinger, 2009), risk of developing AD (Arab & Sabbagh, 2010; Gu, Luchsinger, Stern & Scarmeas, 2010; Scarmeas, Luchsinger, Schupf, Brickman, Cosentino, Tang & Stern, 2009), and mortality from AD (Scarmeas, Luchsinger, Mayeux & Stern, 2007).

**Curcumin, Ginko Biloba, and Caffeine**

Several spices and teas that are typically found in eastern diets are high in anti-inflammatory properties that exhibit neuroprotection against OS induced damage and attenuate neuronal degradation. In AD cell culture and mouse models, treatment with curcumin (turmeric) resulted in a reduction of Aβ 40 and Aβ 42 peptide levels (Zhang, Browne, Child & Tanzi, 2010) and protection against DNA damage (Thomas et al., 2009). Ginko biloba extract reduced inflammation (Boghdady, 2013), thereby up-regulating mitochondrial DNA and reducing Aβ oxidation (Rhein et al., 2010). The polyphenols found in green tea may also improve mitochondrial function (Chen, Lin, Liu & Lin-Shiau, 2008), delay neuronal damage (Park et al., 2009), reduce glial swelling (Panickar, Polansky & Anderson, 2009), and reduce oxidative damage (Etus, Altug, Belce & Ceylan, 2003). Caffeine may also provide neuroprotection against
neurodegenerative diseases as well. Acute administration of caffeine before brain injury significantly reduced Aβ plasma levels in both young and old AD (APP<sub>SW</sub>) mice (Cao et al., 2009). More specifically, higher levels of caffeine in plasma were associated with lower Aβ plasma levels (Cao et al., 2009).

**Vitamins C and E**

Vitamin C and E are naturally occurring antioxidants that also have been shown to improve cognitive deficits and ameliorate AD pathology. In fact, supplementation of vitamins may improve cognitive outcomes in adults with AD, who typically have lower plasma levels of vitamins C and E (Riviere, Birlouez-Aragon, Nourshashemi & Vellas, 1998). In an AD mouse model (APP/PS1), both middle aged and older mice injected with vitamin C one hour prior to behavioral testing showed improvement in spatial learning and memory tasks (Harrison, Hossenini, McDonald & May, 2009). Treatment with vitamin E may also reduce amyloid deposition and behavioral deficits. In another AD mouse model (Tg2576), 11-month-old female mice were given vitamin E four weeks prior to repetitive concussive brain injury, and continued with the supplemented treatment up to eight weeks post-injury (Conte et al., 2004). Injured mice on the supplemented treatment improved in learning and memory tasks and also had reduced amyloid deposition both in the hippocampus and cortex (Conte et al., 2004).

**Berries**

A variety of different berries have also been found to protect against inflammation and neuronal damage caused by AD. In a CO7 cell culture model, blueberries protected
against toxicity from Aβ, leading to better cell viability (Joseph, Fisher & Carey, 2004). Similarly, treating primary mouse cultured microglia with blueberries reduced inflammation and microglial activation (Zhu, Bickford, Sanberg, Giunta & Tan, 2008).

Blueberry supplementation (2% concentration) for 4 months also lowered oxidative stress induced inflammation in older mice (Goyarzu et al., 2004). In addition, AD mice (APP/PS1) given blueberry in rodent chow (2% concentration of blueberry extract milled into pellets) given for 8 months, showed significant improvement in behavior and trended towards lower Aβ plaque burden in cortical tissue (Joseph et al., 2003). Similarly, diets supplemented with blackberries and strawberries also improved age-related deficits in normal aging rats (Joseph et al., 1998; Shukitt-Hale, Cheng & Joseph, 2009).

**Pomegranate Juice**

Pomegranates are naturally high in polyphenolic properties and have been shown to have antioxidant, anti-inflammatory, and anti-apoptotic properties (Dorsey & Greespan, 2000; Ismail, Sestili, & Akhtar, 2012; Seeram et al., 2005; Vegara et al., 2014). Antioxidants found in pomegranates have been found to be neuroprotective in mice with AD pathology (Tg2576) (Hartman et al., 2006). In this study, Hartman used a mere 1:20 diluted concentration of pomegranate juice and found significant behavioral and neuronal improvements. Mice that consumed the pomegranate-enriched water for 6 months improved in spatial learning tasks and had significant reductions in hippocampal Aβ42 and fibrillar Aβ deposition. In another study, maternal supplementation with pomegranate juice influenced mice pup recovery after brain injury (Loren, Seeram, Schulman & Holtzman, 2005). Pregnant mice (C57BL6) received drinking water
containing high, moderate, or low doses of pomegranate juice concentrate. Hypoxic (oxygen deprivation) brain injury was induced 7 days after pups were born and sacrificed at 14 days old. Pups that were exposed to pomegranate juice neonatally, regardless of dose, had less tissue loss than controls, demonstrating the neuroprotective properties of pomegranate polyphenols. Another study also found that pomegranate juice supplementation ameliorated radiation induced depression-like behavior and improved motor performance in male mice (Dulcich & Hartman, 2013). In this study, Dulcich found the 1:20 dilution of juice that was given for 6 weeks to be extremely effective in irradiated mice.

**Grape Polyphenols**

Grapes are also high in polyphenolic properties and have been shown to have antioxidant and anti-inflammatory properties (Azachi, Yatuv, Katz, Hagay, & Danon, 2014; Castilla et al., 2006; Greenspan et al., 2005; Marchi, Paiotti, Artigiani, Oshima, & Ribeiro, 2014; O’Byrne, Devaraj, Grundy, & Jialal, 2002; Singh, Agrawal, & Doré, 2013). Several researchers have also focused on the beneficial effects prompted by the consumption of grapes and its derivatives (polyphenols, flavonoids, anthocyanins, and resveratrol) to ultimately reduce neuronal dysfunction and improve brain health.

Darker varietals of grapes have higher polyphenolic content in their skin and seeds and are commonly used to produce wine and grape juice. Wine has been found to be neuroprotective in mice (Tg2576) with AD neuropathology (Wang et al., 2006). In this study, Wang found that consumption of cabernet sauvignon wine for 7 months lead significant improvements in spatial memory and reduced the overall Aβ plaque burden in
the hippocampus and cortex. In a similar study, cabernet sauvignon treatment for 10 months improved memory and also reduced Aβ peptides in the brains of mice with AD neuropathology (Tg2576) (Ho et al. 2009). In another study, grape juice (Concord) protected against age-related deficits in rats (Schukitt-Hale, Carey, Simon, Mark, & Joseph, 2006). In this study, Schukitt-Hale found that rats given 10% and 50% grape juice for only 2 months improved in motor and learning tasks, respectively. In addition, grapes may also protect against oxidative damage (Ates et al., 2007) and increase hippocampal astrocyte activity (Fujishita et al., 2009). Increased astrocyte activity was also found in cells that were pre-treated with wine (Gomez-Serranillos et al., 2009).

Resveratrol, a polyphenolic compound found in the skin of red grapes is thought to play a significant role in preventing neurotoxicity and inflammation in the brain (Milardi, Stringaro, Colone, Bonincontro, & Risuelo, 2014). Mice pups that were treated with resveratrol 10 minutes and 24 hours prior to hypoxia had fewer inflammatory pathways activated than pups that were given resveratrol 3 hours post-injury (West, Atzeva & Holtzman, 2007). In another study, resveratrol significantly reduced inflammation and microglial activation in the hippocampus and cortex resveratrol when mice were injected 5 minutes and 12 hours post-TBI surgery (Gatson et al., 2012). In addition, resveratrol has also been found to influence AD neuropathology. For example, resveratrol prevented Aβ aggregation, reduced oxidative species, and improved spatial memory in rats (Huang, Lu, Wo, Wu & Yang, 2011). Pre-treatment with resveratrol also led to stronger cell communication and viability in rat hippocampal slices that had been previously exposed to oxidative damage (Almeida et al., 2008). Resveratrol treatment also influenced AD neuropathology in mice. Mice (Tg19959; cross of APP695 with
KM670/671NL and V717F) that received resveratrol had a reduction in both diffuse and fibrillar Aβ pathology in the medial cortex, striatum, and the hypothalamus (Karuppagounder et al., 2009). Similarly, human embryonic kidney 293 cells that were transfected with human APP695 for 2 days and incubated with resveratrol for 24 hours had more Aβ peptide degradation than matched controls (Marambaud, Zhao & Davies, 2005).

Grape seed extract (GSE) is derived from whole grape seeds and contain a concentrated amount of vitamin E, flavonoids, and other polyphenols is also high in antioxidant and anti-inflammatory properties (Boghdady, 2013; Cai et al., 2011; Johnson-Varghese, Brodsky, & Bhandari, 2004; Singh et al., 2013). The neuroprotective effects of GSE have been found to inhibit Aβ formation (Ono et al., 2008) and reduce both Aβ induced toxicity and reactive oxidative species (Li, Jang, Sun & Surh, 2004) in cultured cells. These findings have also been translated into AD animal models. In one study, mice (Tg2576) that consumed GSE (200 mg/kg/day) for 5 months had lower levels of reactive Aβ oligomeric species and showed improvement in spatial learning memory tasks (Wang et al., 2008). In another study, mice (PSAPP) that consumed a GSE enriched diet of 592.5 g/d over the course of 9 months had significant reductions in Aβ plaque deposition in the neocortex and hippocampus, in addition to lower levels of microglial activity (Wang et al., 2009). Grape polyphenol extract was also found to ameliorate injury induced oxidative and neuronal damage (Wang et al., 2009).

Diets enriched with high polyphenolic compounds can have a strong non-pharmaceutical impact in attenuating cognitive decline and neurological diseases. Grapes and its derivatives have shown several neuroprotective effects resulting from the
antioxidant and anti-inflammatory properties in the fruit. However, whether the inclusion of different types of grapes can prevent or slow down the combination of AD-related pathology and brain injury is not clear. In this study, we investigated the impact of a high polyphenol grape-enriched diet on cognitive and neuronal degeneration caused in PSAPP mice and brain injury.

**Aims of the Current Study**

This study aimed to explore the potential neuroprotective effects of a high polyphenol grape diet in transgenic PSAPP mice and its influence on attenuating brain injury.

- **Aim 1**: To determine whether a grape-enriched diet reduced lesion size from brain injury.
  - **Hypothesis 1**: Mice on the grape diet will have smaller lesions than mice on the control diet (main effect of diet).

- **Aim 2**: To determine whether a grape-enriched diet and/or brain injury influenced behavior.
  - **Hypothesis 2a**: Mice on the grape diet will have fewer behavioral deficits than mice on the control diet (main effect of diet).
  - **Hypothesis 2b**: Mice with brain injury will have more behavioral deficits than control mice (main effect of injury).

- **Aim 3**: To determine whether a grape-enriched diet and/or brain injury influenced AD neuropathology
Hypothesis 3a: Mice on the grape diet will have lower Aβ levels than mice on the control diet (main effect of diet).

Hypothesis 3b: Mice with TBI will have more Aβ than naïve and sham mice (main effect of TBI).

Hypothesis 3c: TBI mice on the grape-enriched diet will have smaller lesions and less Aβ deposition than TBI mice on the control diet (diet x TBI interaction).

Aim 4: To determine whether a grape diet and/or brain injury influenced BBB clearance and astrogliosis.

Hypothesis 4a: Mice on the grape diet will have less RAGE and astrogliosis reactivity than mice on the control diet (main effect of diet).

Hypothesis 4b: Mice with TBI will have more RAGE and astrogliosis reactivity than naïve and craniotomy mice (main effect of TBI).

Hypothesis 4c: TBI mice on the grape-enriched diet will have less RAGE and astrogliosis than mice on the control diet (diet x TBI interaction).
CHAPTER TWO

METHODS

Experimental Design

Male and female mice (3 to 5 months old) were separated into 6 groups (diet/no-diet, craniotomy/control, TBI/control), yielding a total of 12 groups (Table 1). Thirty-one animals were started on the grape-enriched diet immediately and continued on this diet until the time of sacrifice. The grape-enriched diet was packed into rodent pellets. Thirty-one animals did not receive the grape rodent pellets, but received control sugar pellets.

Table 1

*Projected animal group breakdown.*

<table>
<thead>
<tr>
<th></th>
<th>Grape-Enriched</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>Male: n=8</td>
<td>Male: n=5</td>
</tr>
<tr>
<td></td>
<td>Female: n=3</td>
<td>Female: n=4</td>
</tr>
<tr>
<td>Craniotomy</td>
<td>Male: n=6</td>
<td>Male: n=5</td>
</tr>
<tr>
<td></td>
<td>Female: n=3</td>
<td>Female: n=6</td>
</tr>
<tr>
<td>CCI</td>
<td>Male: n=6</td>
<td>Male: n=5</td>
</tr>
<tr>
<td></td>
<td>Female: n=5</td>
<td>Female: n=5</td>
</tr>
</tbody>
</table>

Animals were allowed to acclimate for 4 weeks before surgeries (anesthesia control, craniotomy, or CCI). After surgery, animals (craniotomy and CCI) underwent magnetic resonance imaging (MRI). Thereafter, all animals were tested a battery of
behavioral tests for 2 weeks. The tests assessed cognitive, motor, and learning abilities. After a total of 8 weeks, mice were perfused with paraformaldehyde via the left ventricle with saline until euthanization. Once euthanization was confirmed, the animals’ brains were removed and placed in paraformaldehyde for 24 hours, then washed in saline, frozen with dry ice, and stored at -20 degrees Celsius until sectioning. See Figure 2 for experimental timeline.

**Experimental Design Timeline**

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 8</th>
<th>Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice arrive</td>
<td>Surgery</td>
<td>MRI</td>
<td>Behavioral Testing</td>
<td>Perfusions</td>
<td>Tissue Processing</td>
</tr>
<tr>
<td>Control &amp; grape-enriched diets began</td>
<td>• Craniotomy • Controlled Cortical Impact</td>
<td>• Craniotomy • Controlled Cortical Impact</td>
<td>• Open Field • Rotarod • Zero Maze • Water Maze • Tail Suspension Test</td>
<td>All mice</td>
<td>All mice Histology &amp; immunohisto-chemistry protocols</td>
</tr>
</tbody>
</table>

*Figure 2.* Timeline of the experimental design.

**Animals**

Sixty-one PSAPP (double transgenic mice exhibiting pathology similar to AD) mice between 3 to 5 months in age were obtained from Jackson Laboratories (Bar Harbor, ME) and divided into 4 groups (TBI vs. craniotomy controls, grape vs. none). Mice received either a control or grape-enriched diet for 8 weeks. The grape-enriched diet mice consumed a daily average of 0.5 mg of polyphenols from the grape powder in their food (15.5% by weight). The control diet contained sugars matching the caloric
context of the sugars in the grape diet (14% by weight). Previous research from our lab has shown 0.3-0.6 mg per day of dietary pomegranate polyphenols decreased Aβ levels and plaque load and improved behavior in Tg2576 mice (Hartman, et al., 2006). Four craniotomy and 6 CCI mice died before the completion of the study from injury related complications (See Table 2 for final group numbers). There were no significant weight differences between the groups.

Table 2

*Final animal group breakdown.*

<table>
<thead>
<tr>
<th></th>
<th>Grape-Enriched</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naïve</strong></td>
<td>Male: n=8</td>
<td>Male: n=5</td>
</tr>
<tr>
<td></td>
<td>Female: n=3</td>
<td>Female: n=4</td>
</tr>
<tr>
<td><strong>Craniotomy</strong></td>
<td>Male: n=4</td>
<td>Male: n=3</td>
</tr>
<tr>
<td></td>
<td>Female: n=3</td>
<td>Female: n=6</td>
</tr>
<tr>
<td><strong>CCI</strong></td>
<td>Male: n=2</td>
<td>Male: n=2</td>
</tr>
<tr>
<td></td>
<td>Female: n=4</td>
<td>Female: n=3</td>
</tr>
</tbody>
</table>

**Grape-Enriched Diet**

The California Table Grape Commission provided the freeze-dried grape powder. Red, green, and blue-black seeded and seedless grape varieties were used. These were frozen, ground with food-quality dry ice, freeze-dried, and then re-ground. The dry powder contained approximately 1% moisture and was stored at -70ºC in moisture-proof
containers. The compounds in the dry powder were similar as those found in fresh grapes such as catechins, anthocyanins, flavinols, and resveratrol. The grape powder was milled into Harlan Teklad 8640 High Protein Rodent Diet pellets (standard pellet is 3.2 Kcal/g). The grape-enriched diet had 15.5% (by weight) grape powder added to the pellet which included 18.3% of protein (22.9% of kcal), 51.4% of carbohydrates (64.4% of kcal), and 4.5% of fat (12.7% of kcal). The control diet had 14% (by weight) sucrose added to the pellet which included 18.6% of protein (23.5% of kcal), 50.5% of carbohydrates (63.7% of kcal), and 4.5% of fat (12.7% of kcal).

**Traumatic Brain Injury Induction**

After 4 weeks of dietary treatment, 20 animals underwent traumatic brain injury (controlled cortical impact) or sham surgery (TBI vs. sham, TBI with diet vs. TBI control diet), followed by two weeks for recovery. Surgeries were preformed as described in Brody et al. 2007. Briefly, animals were anesthetized with 3% isoflurane and local anesthetic (intradermal injection of lidocaine HCl 2% + 1:100,000 epinephrine). Following a midline incision, skim overlying the skull was retracted. A 5 mm craniotomy was induced in the right hemisphere (immediately adjacent to the Sagittal midline and 4.0 mm anterior to Bregma). The heads of the mice were adjusted to allow the craniotomy to penetrate the thin skull without damaging the dura surrounding the brain. CCI was induced using an electromechanical impactor using a 3 mm tip. The CCI was delivered at a depth of 2.5 mm at a velocity of 5 m/s into the cortex; with a dwell time of 100 ms. After several mice began to die shortly following CCI, the depth of the injury was readjusted to 1.5 mm, with all other parameters remaining as before. Craniotomy mice
underwent similar surgery conditions with the exception that the impact tip only touched the dura matter, rather than directly impacting the cortex.

**Behavioral Testing**

After 6 weeks of dietary treatment, all mice underwent a 2-week battery of behavioral tests to assess cognitive and motor abilities. Dietary treatment continued throughout testing until sacrifice.

**Open Field Activity**

General activity levels and movement patterns were assessed with an open field activity test, which involves the observation of an animal for 30 minutes in opaque open-topped plastic boxes (49 x 36 cm). The movements of the animal were recorded with an overhead camera and analyzed by a computerized tracking system (Nodlus Ethovision). Examples of some of the parameters that were analyzed, including distance moved, percent time spent moving, movement velocity, path meander/tortuosity, and the number of rearings.

**Rotarod**

Sensorimotor and coordination were assessed with the accelerating rotarod (Columbus Instruments). This test consists of 4 rotating horizontal cylinders at a diameter of 3 cm. Mice are placed onto the cylinder and have to walk continuously forward to avoid falling. Latency to fall off is the dependent variable; with performance over days of testing is a measure of motor learning. Mice were tested every other day for 3 days. Three
blocks of 2 consecutive trials were administered per day: 2 stationary trials, 2 trials at 5 RPM, and 2 trials that start at 10 RPM and increased by 2 RPM every 2 seconds.

**Elevated Zero Maze**

The elevated zero maze was used to assess exploratory behaviors in an anxiety-provoking environment. The maze consisted of a plastic ring, with a 100 cm outer diameter, 10 cm wide. The two opposing quadrants were enclosed with 35 cm walls. The room was dimmed, and halogen lights directly illuminated the open spaces of the maze. Animals were placed in the center of one of the open spaces and allowed to freely explore the zero maze for 5 minutes. The percentage of time spent in the enclosed quadrants was calculated. Spending time in the enclosed spaces is generally associated with anxiety-like behavior.

**Water Maze**

Learning and memory were assessed with the water maze. This is a test of spatial navigation that requires an animal to learn the location of a hidden platform in a pool of water using the visual cues from around the room. The water maze consists of a large metal pool (110 cm diameter) filled with water that is colored opaque with white tempura paint. The pool contains a moveable platform (11 cm diameter) that the animal can step onto to escape the water. Animals were given a total of 10 trials per day. For each trial, animals were released with their nose against the wall into the pool at one of the 4 release points and were allowed to swim to the platform. The trials lasted a maximum of 60 seconds and mice were manually guided to the platform if they did not find the platform
in time. An overhead camera recorded the swim paths which gathered data for the quantification of distance, latency, proximity to target, and swimming speed by a computerized tracking system (Nodlus Ethovision).

On day 1, animals were given a cued water maze trial, which is a control task for assessing sensorimotor and/or motivational deficits that may affect performance during the spatial phase. The surface of the escape platform was visible (5 mm above the surface of the water) and a pole was placed on top of the platform to make its location more obvious. The location of the platform varied from trial to trial. Animals were released into the pool opposite the location of the platform and were allowed to remain on the platform for 5 seconds after finding it.

On days 2-5, animals were given a spatial water maze, in which the mice were to find the platform based on its relationship to the spatial cues around the room, rather than direct visualization. The escape platform was submerged 1 cm below the surface of the opaque water and the location of the platform changed each day for 3 days. After finding the platform, animals were allowed to remain on there for an additional 5 seconds. The next day, animals were given a probe trial in which the platform was removed from the pool, and the animal was allowed to search for the platform for 60 seconds. Amount of time the animal spent in the probe quadrant was measured as well as the total number of times the animal crossed over the former location of the platform. An hour later, the platform was placed back into the pool at a new location, and the next sets of 10 trials were administered.
**Tail Suspension Test**

The tail suspension test was administered to assess depression-like behaviors. Mice were suspended by the tail with adhesive tape that was attached approximately 1 cm from the tip of the tail. The other end of the tape was wrapped around a hook that was embedded in the center of the ceiling of a wooden box (19 cm x 21 cm x 40 cm). Once suspended, the animal’s rostral end was approximately 20 cm from the floor of the box. The box was enclosed on all sides except for the viewing side, and lighting and sound in the room were kept at a minimum. Each animal received one 6-min trial and was rated on mobility and agitation for the duration of 6 minutes. The time that the animal remained immobile during the final 4 minutes of the trial was recorded. Immobility was defined as the complete lack of movement by the mouse, even if it was still swinging back and forth from a previous struggle or if it was curled up while holding its paws (as long as it was not struggling or moving otherwise).

**Tissue Preparation**

After eight weeks of dietary treatment, mice were perfused through the heart with phosphate buffered saline (PBS), and brains were extracted. The right hemisphere of each brain was immediately removed, frozen at -80°C and stored for future biochemical analyses. In preparation for histology and immunohistochemistry, the left hemisphere of each brain \((n = 48)\) were immersed in 4% paraformaldehyde in 0.1 M PBS solution and stored at 4°C. Thereafter, the brain tissue was fixed in a 30% sucrose solution at 4°C for 72 hours, followed by freezing on dry ice. The left hemispheres were cut as coronal cryostat sections (20 μm) at -20°C from the frontal cortex to the cerebellum (Fisher;
Leica CM1950; Leica Microsystems GmbH, Wetzlar, Germany). Brain tissue was directly mounted on glass slides (Gold Plus, Fisher) and stored at -20°C. Sectioning resulted in 50 slides per animal with 6-8 sections per slide. Each slide was a representation of the animal’s entire brain.

**Staining and Quantification Protocols**

**Histology**

Before beginning the staining protocol for each experiment, slides were removed from storage and were acclimated to room temperature (~20 minutes). Thereafter, each slide was placed on a warmer at 38°C for 1 hour to ensure sections adhered to the slides well. Then slides were quickly dipped in double distilled water (ddH₂O) and were dried at room temperature for an additional 3 hours to prevent brain tissue from floating off during the protocol.

**Thioflavin-S**

Slides were stained (20 naïve, 14 craniotomy, 10 CCI) with thioflavin-S to detect fibrillar Aβ, as previously described (DeMattos et al., 2002). Thioflavin-S was removed from 4°C to acclimate to room temperature. Slides were washed in 3 consecutive 5-minute washes of phosphate buffered saline (PBS; pH 7.4; Fisher Scientific, Pittsburgh, PA) to remove any mounting medium surrounding the brain sections. After, slides underwent two 5-minute washes of ddH₂O to acclimate brain sections to ddH₂O because thioflavin-S is water based. The following procedures were completed inside a hood in the dark to keep the stain’s fluorescent properties intact. Slides were stained in 1%
thioflavin-S solution for 5 minutes, followed by washes in 70% ethanol (EtOH) 2 x 3 minutes and 90% EtOH 1 x 3 minute. Ethanol steps were used to increase the differentiation of the stain and to lower background fluorescence. Brain tissue from 2 naïve and 1 sham slides floated off during the staining protocol and could not be used for analysis. Once sections were dry, slides were cover-slipped with anti-fading medium (Vectashield; Vector Laboratories, Burlingame, CA) containing 4,6-diamino-2-phenylindole (DAPI), which stained cell nuclei. Slides were sealed with clear nail polish and were stored in a slide box at 4˚C for analysis.

**Quantification of Thioflavin-S**

Quantification of fibrillar Aβ plaque load was assessed using the Mercator software (Explora-Nova, La Rochelle, France) with an epifluorescence microscope (Olympus, BX41, Center Valley, PA USA). All of the analytical procedures were completed in the dark to protect the fluorescent properties of the stain. Slides were removed from storage one at a time and were cleaned with 70% EtOH on Kim wipes to remove any dust or fingerprints. One section per slide (Bregma level ~ -1.94 mm; Figure 2B) was analyzed to ensure consistency among results. The hippocampus and dorsal cortex regions were manually outlined, as regions of interest and the threshold and user-defined parameters were kept consistent for all animals. Once completed, false positives (i.e. vessels or dust) were double checked and manually deleted in the hippocampus and dorsal cortex regions. Using the region statistics that were automatically generated in the program, the % fibrillar Aβ plaque load was calculated.
Quantification of Brain Area

Slides stained with thioflavin-S were used to quantify brain area with the Mercator software (Explora-Nova, La Rochelle, France) on the microscope. Brain sections at Bregma level -1.94 mm on each slide were outlined. In addition, the ventricles and open spaces on the brain section were outlined and subtracted from the brain area.

Immunohistochemistry

For all the immunohistochemistry experiments, slides were removed from storage at -20°C to acclimate to room temperature. Once acclimated (~20 minutes), each slide was placed on a warmer at 38°C for 1 hour to ensure sections adhered well. Slides were quickly dipped in ddH2O and were dried at room temperature for an additional 3 hours to prevent brain tissue from floating off during washes and staining.

Monoclonal HJ3.4 Amyloid Beta Antibody

Slides were stained (20 Naïve, 14 craniotomy, 12 CCI) with the HJ3.4 antibody to detect all confirmations of Aβ, (kindly provided by John Cirrito at the Washington University, St. Louis, MO). This antibody binds to the central domain of Aβ and has a secondary fluorescent tag already attached to the primary. Slides underwent washes of PBS 2 x 10 minutes, followed by outlining the tissue with a Dako pen to create a boundary for the antibody to stay within. Brain sections were blocked at room temperature in a humid chamber for 90 minutes in 1% bovine serum albumin (BSA) in PBS (Sigma-Aldrich Co., St. Louis, MO). Next, slides were incubated overnight at 4°C with HJ3.4 antibody (mouse, 1:1000; Washington University School of Medicine, St.
Louis, MO) in 0.25% BSA/0.25% TritonX-100 in PBS (Sigma-Aldrich Co., St. Louis, MO). The next day, slides were rinsed in PBS 2 x 10 minutes, followed by washes in ddH$_2$O 2 x 1 minute to remove PBS salts. Once sections were dry, they were cover-slipped with Vectashield containing DAPI, sealed with clear nail polish, and stored in a slide box at 4°C for analysis.

To further optimize staining, a formic acid (88%) antigen retrieval technique was used. Slides underwent washes of PBS 2 x 10 minutes, followed by outlining the tissue with a Dako pen to create a boundary for the formic acid to stay within. Formic acid was added to cover each tissue section (~2 drops) for 4 minutes. Slides were immediately washed with PBS to stop formic acid activity, followed by 3 x 10 minutes of PBS washes. Brain sections were blocked at room temperature in a humid chamber for 90 minutes in 1% BSA in PBS (Sigma-Aldrich Co., St. Louis, MO). Next, slides were incubated overnight at 4°C with HJ3.4 antibody (mouse, 1:1000; Washington University School of Medicine, St. Louis, MO) in 0.25% BSA/0.25% TritonX-100 in PBS (Sigma-Aldrich Co., St. Louis, MO). The next day, slides were rinsed in PBS 2 x 10 minutes, followed by washes in ddH$_2$O 2 x 1 minute to remove PBS salts. Once sections were dry, they were cover-slipped with Vectashield containing DAPI, sealed with clear nail polish, and stored in a slide box at 4°C for analysis. There were no significant differences in staining between tissues that underwent formic acid treatment to those that did not receive antigen retrieval.

In another attempt to optimize staining, a citrate buffer (10mM citric acid, 0.05% tween 20, pH 6.0) antigen technique was used. Slides underwent washes of PBS 2 x 10 minutes, and placed in the citrate buffer solution. Slides in the buffer solution were
heated in the microwave for 5 minutes and were allowed to cool to room temperature. Once cooled, slides underwent 2 x 10 minutes PBS washes, followed by outlining the tissue with a Dako pen to create a boundary for the blocking solution. Brain sections were blocked at room temperature in a humid chamber for 90 minutes in 1% BSA in PBS (Sigma-Aldrich Co., St. Louis, MO). Next, slides were incubated overnight at 4°C with HJ3.4 antibody (mouse, 1:1000; Washington University School of Medicine, St. Louis, MO) in 0.25% BSA/0.25% TritonX-100 in PBS (Sigma-Aldrich Co., St. Louis, MO). The next day, slides were rinsed in PBS 2 x 10 minutes, followed by washes in ddH₂O 2 x 1 minute to remove PBS salts. Once sections were dry, they were cover-slipped with Vectashield containing DAPI, sealed with clear nail polish, and stored in a slide box at 4°C for analysis. There were no significant differences in staining between tissues that underwent citrate buffer treatment to those that did not receive antigen retrieval.

**Quantification of HJ3.4**

Quantification of global Aβ plaque load was assessed using the Mercator software (Explora-Nova, La Rochelle, France) with an epifluorescence microscope (Olympus, BX41, Center Valley, PA USA). All of the analytical procedures were completed in the dark to protect the fluorescent properties of the stain. Slides were removed from storage one at a time and were cleaned with 70% EtOH on Kim wipes to remove any dust or fingerprints. One section per slide (Bregma level ~1.70 mm, ~-1.94 mm, and ~-3.52 mm; Figure 3) was analyzed to ensure consistency among results. The hippocampus and dorsal cortex regions were manually outlined, as regions of interest and the threshold and user-defined parameters were kept consistent for all animals. Once completed, false
positives (i.e. vessels or dust) were double checked and manually deleted in the hippocampus and dorsal cortex regions. Using the region statistics that were automatically generated in the program, the % Aβ plaque load was calculated.

**Figure 3.** Bregma levels in the brain. (A) The entire cortical surface of the brain section at Bregma ~1.70 mm was analyzed for global Aβ plaque load. (B) The hippocampus and the overlying dorsal cortical surface of the brain section at Bregma ~ -1.94 mm was analyzed for global Aβ and fibrillar Aβ plaque load. (C) The cortical surface immediately lateral to the hippocampus at Bregma ~ -3.52 mm was analyzed for global Aβ plaque load.

**Glial Fibrillary Acidic Protein Antibody**

Glial fibrillary acidic protein (GFAP) antibody was used to assess the astroglial immunoreactivity in the dorsal parietal cortex. Expressed in the central nervous system, GFAP is an intermediate filament protein that is believed to help maintain astrocyte mechanical strength and shape expressed in astrocytes. Twenty naïve, 14 craniotomy, and 12 CCI slides were stained. Slides underwent washes in PBS 2 x 10 minutes, followed by outlining the tissue with a Dako pen. Brain sections were blocked at room temperature in
a humid chamber for 90 minutes in 1% BSA in PBS. Next, slides were incubated overnight at 4ºC with the primary (1º) antibody (GFAP; chicken, 1:1000, Millipore, Massachusetts) in 0.25% BSA/0.25% TritonX-100 in PBS. The next day, slides were rinsed in PBS 2 x 10 minutes, followed by incubation in a humid chamber with the secondary (2º) antibody (anti-chicken, 1:1000, Odyssey, coupled with Alexa-Flur-680 for infra-red analysis) in 0.25% BSA/0.25% TritonX-100 in PBS at room temperature for 90 minutes. To decrease background and remove excess 2º, slides were rinsed in PBS 3 x 10 minutes. After, slides were rinsed in ddH2O 2 x 1 minute to remove the PBS salts. Once sections were dry, they were cover-slipped with Vectashield containing DAPI, sealed with clear nail polish, and stored in a slide box at 4˚C for analysis. Brain tissue from 2 naïve and 2 sham slides floated off during the staining protocol and could not be used for analysis.

**Receptor for Advanced Glycation Endproducts Antibody**

Receptor for advanced glycation endproducts (RAGE) labeling will be used to measure the influx of peripheral Aβ peptides across the BBB into the brain. Twenty naïve, 14 craniotomy, and 12 CCI slides were stained. Slides were washed in PBS 2 x 10 minutes, followed by outlining the tissue with a Dako pen. Brain sections were blocked at room temperature in a humid chamber for 90 minutes in 1% BSA in PBS. Next, slides were incubated overnight at 4ºC with the 1º antibody (RAGE; rabbit, 1:1000, Abcam, Massachusetts) in 0.25% BSA/0.25% TritonX-100 in PBS. The next day, slides were rinsed in PBS 2 x 10 minutes, followed by incubation in a humid chamber with the 2º antibody (anti-rabbit, 1:1000, Rockland Immunochemicals Inc., PA) coupled with Alexa-
Flur-800 for infra-red analysis) in 0.25% BSA/0.25% TritonX-100 in PBS at room temperature for 90 minutes. To decrease background and remove excess 2°, slides were rinsed in PBS 3 x 10 minutes. In addition, slides went through washes in ddH$_2$O 2 x 1 minute to remove the PBS salts. Once sections were dry, slides were cover-slipped with Vectashield containing DAPI, sealed with clear nail polish, and stored in a slide box at 4°C for analysis. Brain tissue from 2 naïve and 2 sham slides floated off during the staining protocol and could not be used for analysis.

**Quantification of GFAP and RAGE**

The Licor-Odyssey scanner detects the secondary antibodies by recording the intensity of the infrared signal through a photomultiplier. The photomultiplier is used to precisely detect light by multiplying the current produced by a light millions of times, allowing individual photons to be detected and visible on an image. The intensities of the cytoplasm of the neurons were analyzed using the red (700 nm) and green (800 nm) channels. Each slide was scanned into the program using the same parameters for all the brain tissue at 21μm/pixel resolution, 3.0 intensity for GFAP, and 6.0 intensity for RAGE (Licor Bio-Science, Lincoln, NE). The averaged intensity (I.I.) scores were obtained from five independent fields-of-interest drawn close to one another with the Licor-Odyssey analysis software on the cortex directly adjacent to the hippocampus (Bregma level ~ - 1.94 mm). The specific region of the dorsal parietal cortex was chosen for because this area was present on all the animals, in contrast to the hippocampus. Pixel intensities ranged from 0 (dark) to 255 (light), in which lower pixel intensities represented lower density and immunoreactivity.
**Statistical Analysis**

IBM SPSS 21 was used to analyze data. An α-level of 0.05 was used for all statistical significance tests. Correlations between behavioral, histology, and immunohistochemistry variables were determined using the Pearson product-moment coefficient. Data from all behavioral testing, histology and immunohistochemistry staining were analyzed with one-way analysis of variances (ANOVAs; group: control vs. grape, injury, gender). Two-way ANOVAs with two between-subjects factors were used to further distinguish any group interactions. Swim data from the water maze was analyzed by averaging trials into 5 blocks of 2 trials each. These blocks were analyzed with repeated measure ANOVAs that included one within-subjects variable (test day). Probe swim data were analyzed by averaging all 3 trials. To avoid violating the assumptions of compound symmetry and sphericity, the reported p-values for every repeated-measures analysis reflected the Huynh-Feldt adjustment to the degrees of freedom. Graphs were created using the Prism 6.0 statistical program.

The procedures and necessary equipment described above were available and routinely used by the Badaut laboratory at Loma Linda University.
CHAPTER THREE

RESULTS

Aim 1: Influence of a Grape-Enriched Diet on Lesion Size

Structural MRI data revealed that TBI mice ($M = 5.79$, $SD = 1.78$) produced a larger brain injury than craniotomy mice ($M = 1.81$, $SD = .94$), $F(1, 26) = 58.82$, $p < .01$; Figure 3A. There was no overall effect of diet on brain lesion ($p > .05$; Figure 4B). Craniotomy mice on the enriched diet had smaller and more consistent lesions than control, though results did not reach statistical significance ($p > .05$; Figure 4C).

Figure 4. Percentage of lesion volume was determined using structural MRI data. (A) TBI in mice resulted in larger percentages of brain lesioned than those with craniotomy ($p < .01$). (B) Overall, grape-enriched diet did not influence the percentage of brain lesioned ($p > .05$). (C) Grape-enriched craniotomy had smaller percentages of brain damage compared to control, though these results did not reach statistical significance ($p > .05$).
Aim 2: The Influence of a Grape-Enriched Diet and/or Brain Injury on Behavior

Influence of Diet on Behavior

General exploratory behaviors were assessed in open field testing. Overall, diet did not influence hyperactivity or exploratory behaviors in open field testing ($p > .05$; Figure 5, 6).

![Figure 5](image)

Figure 5. Diet did not influence general activity levels. (A) The enriched diet did not affect the percentage of time mice spent moving during the test ($p > .05$). (B) The enriched diet did not affect the distance mice traveled ($p > .05$). (C) The enriched diet did not affect the velocity of mice during exploration ($p > .05$). (D) Diet did not influence the percentage of time spent in the perimeter of open field box ($p > .05$).
Figure 6. Diet did not influence general activity levels. (A, B) Diet did not affect the percentage of time spent exploring in the center or corner of the open field test ($p > .05$).

Sensorimotor and coordination were tested with the rotarod. Overall, the grape-enriched diet did not improve performance on the rotarod ($p > .05$; Figure 7). Performance did not improve on day 2 of testing in either the steady or accelerating rotarod trials ($p > .05$; Figure 7).
Figure 7. (A, B) The grape-enriched diet did not influence motor performance on the steady trials of the rotarod across both days of testing ($p > .05$). (C, D) The grape-enriched diet did not influence motor performance on the accelerating rotarod trials across both days of testing ($p > .05$).

However, craniotomy mice on the grape-enriched diet showed significant behavioral improvements. Grape-enriched mice stayed on the more difficult accelerating and faster rotarod trials longer on day 1 of testing, though results did not reach statistical significance (Figure 8).
Figure 8. Grape-enriched diet influenced motor behavior in craniotomy mice. (A) Craniotomy mice on the enriched diet had a longer latency to fall of the rotarod, though; results did not reach statistical significance ($p > .05$). (B) Craniotomy mice on the enriched diet had a longer latency to fall off the rotarod during the most difficult trial, however, these results did not reach statistical significance ($p > .05$).

The grape-enriched diet did not influence motor performance on the fast, more challenging, rotarod trail across days of testing ($p > .05$; Figure 9).

Figure 9. Diet did not influence motor coordination. (A, B) The grape-enriched diet did not affect overall motor performance on the fastest trials of the rotarod ($p > .05$).

Anxiety-like behaviors were assessed with the elevated zero maze. Diet did not affect the time animals spent in the dark portion of the maze on both days of testing ($p > .05$; Figure 10). In addition, performance did not change on day 2 ($p > .05$).
Figure 10. Diet did not improve anxiety-like behaviors. (A) The grape-enriched diet did not influence the percentage of time mice spent in the dark on day 1 of the zero maze ($p > .05$). (B) The grape-enriched diet did not influence the percentage of time mice spent in the dark on day 2 of the zero maze ($p > .05$). (C) Overall, the enriched diet did not affect the average time spent in the dark in the zero maze ($p > .05$).

Learning and memory were assessed with the water maze. Diet did not affect learning and memory deficits in the cued water maze trials ($p > .05$; Figure 11).
Figure 11. Diet did not influence performance on the cued water maze. (A, C, D) The grape-enriched diet did not affect the distance traveled in the water maze ($p > .05$). (B) The grape-enriched diet did not influence the time in locating the platform in the water maze ($p > .05$).

Similarly, diet did not affect the distance moved (Figure 12), time to platform (Figure 13), cumulative distance to the platform (Figure 14), average distance to the platform (Figure 15), swim speed (Figure 16), and turn bias (Figure 17) in the spatial water maze ($p > .05$).
Figure 12. Diet did not affect spatial water maze performance. (A) The grape-enriched diet did not affect the distance mice moved in the spatial 1 water maze ($p > .05$). (B) The grape-enriched diet did not affect the distance mice moved in the spatial 2 water maze ($p > .05$). (C) The grape-enriched diet did not affect the distance mice moved in the spatial 3 water maze ($p > .05$).
Figure 13. Diet did not affect the time it mice took to find the submerged platform in the water maze. (A) Grape-enriched mice did not find the submerged platform faster than control in the spatial 1 water maze ($p > .05$). (B) Grape-enriched mice did not find the submerged platform faster than control in the spatial 2 water maze ($p > .05$). (C) Grape-enriched mice did not find the submerged platform faster than control in the spatial 3 water maze ($p > .05$).
Figure 14. Diet did not affect the cumulative distance mice traveled in the water maze. (A) Mice on the grape-enriched diet did not travel a shorter distance compared to control in the spatial 1 water maze ($p > .05$). (B) Mice on the grape-enriched diet did not travel a shorter distance compared to control in the spatial 2 water maze ($p > .05$). (C) Mice on the grape-enriched diet did not travel a shorter distance compared to control in the spatial 3 water maze ($p > .05$). (D) Mice on the grape-enriched diet did not travel a shorter distance in the averaged probe trials in the water maze ($p > .05$).
Figure 15. Diet did not affect the average distance traveled to the submerged platform in the water maze. (A) Mice on the enriched-grape diet did not travel a shorter distance to the platform than control in the spatial 1 water maze ($p > .05$). (B) Mice on the enriched-grape diet did not travel a shorter distance to the platform than control in the spatial 2 water maze ($p > .05$). (C) Mice on the grape-enriched diet did not travel a shorter distance to the platform than control in the spatial 3 water maze ($p > .05$). (D) Mice on the grape-enriched diet did not travel a shorter distance to the platform than control in the probe water maze trials ($p > .05$).
Figure 16. Diet did not influence turn bias in the water maze. (A) The grape-enriched diet did not affect turn bias in the spatial 1 water maze ($p > .05$). (B) The grape-enriched diet did not affect turn bias in the spatial 2 water maze ($p > .05$). (C) The grape-enriched diet did not affect turn bias in the spatial 3 water maze ($p > .05$). (D) The grape-enriched diet did not affect turn bias in the probe water maze ($p > .05$).
Figure 17. Diet did not affect swim speed in the water maze. (A) The grape-enriched diet did not affect velocity in the spatial 1 water maze ($p > .05$). (B) The grape-enriched diet did not affect velocity in the spatial 2 water maze ($p > .05$). (C) The grape-enriched diet did not affect velocity in the spatial 3 water maze ($p > .05$). (D) The grape-enriched diet did not affect velocity in the probe water maze ($p > .05$).

Depression-like behaviors were tested with the tail suspension test. Overall, the grape-enriched diet had no affect on depression-like behaviors, regardless of injury ($p > .05$; Figure 18).
Figure 18. Diet did not influence depression-like behaviors. (A) The grape-enriched diet did not affect the amount of time mice spent immobile in the tail suspension test ($p > .05$). (B) The grape-enriched diet did not affect the amount of time mice spent immobile during the last 4 minutes in the tail suspension test ($p > .05$). (C) The grape-enriched diet did not affect the time mice first “gave up” in the tail suspension test ($p > .05$).

However, when looking at TBI alone, diet displayed a significant role in depression-like behaviors. Surprisingly, TBI mice on the enriched diet ($M = 17.58, SD = 4.10$) spent more time immobile on the tail suspension test than those on the control diet ($M = 31.62, SD = 11.03$), $F(1, 8) = 5.75, p < .05$; Figure 19).
Figure 19. Grape-enriched diet influenced depression-like behaviors. (A) The grape-enriched diet did not affect the amount of time all mice spent immobile ($p > .05$). (B) The grape-enriched diet induced depression-like behaviors in TBI mice, where these mice spent more time immobile on the test than controls ($p < .05$).

**The Influence of Injury on Behavior**

Brain injury did not affect general anxiety and exploratory patterns in open field testing ($p > .05$; Figure 20, 21).
Figure 20. Brain injury did not alter general exploratory activity levels. (A) Naive, craniotomy, and CCI mice did not differ in the percentage of time spent moving in open field testing ($p > .05$). (B) Naïve, craniotomy, and CCI mice did not differ in the amount of distance traveled in open field testing ($p > .05$). (C) Naïve, craniotomy, and CCI mice did not differ in velocity in open field testing ($p > .05$). (D) Naïve, craniotomy, and CCI mice did not differ in the percentage of time spent in the perimeter of the open field testing ($p > .05$).

Figure 21. Brain injury did not alter general activity levels. (A) Naïve, craniotomy, and CCI mice did not differ in the percentage of time spent in the center of the test ($p > .05$). (B) Naïve, craniotomy, and CCI mice did not differ in the percentage of time spent in the corner of the test ($p > .05$).
Gender did not influence anxiety and exploratory patterns (Figure 22, 23); however, female mice trended towards exploring the test at a higher velocity than males ($p < .01$; Figure 19C). More specifically, naïve female ($M = 7.04$, $SD = .66$) mice were more hyperactive, exploring the test at a higher velocity than naïve males ($M = 6.41$, $SD = .52$) ($F(1, 18) = 5.53$, $p < .05$; data not shown).

*Figure 22.* General exploratory activity levels were not influenced by gender. (A) Male and female mice did not differ in the percentage of time mice spent moving during open field testing ($p > .05$). (B) Male and female mice did not differ in the amount of distance traveled during open field testing ($p > .05$). (C) Female mice trended towards traveling at a higher velocity during testing than males ($p = .06$). (D) Male and female mice did not differ in the percentage of time mice spent in the perimeter of the test.
Figure 23. General exploratory activity levels were not influenced by gender. (A) Male and female mice did not differ in the percentage of time mice spent in the center of the test ($p > .05$). (B) Male and female mice did not differ in the percentage of time mice spent in the corner of the test ($p > .05$).

Brain injured animals exhibited motor deficits on the rotarod. Although differences were not found on day 1 (Figure 24A, 24C), day 2 of testing revealed injury dependent impaired performance. Craniotomy ($M = 30.77$, $SD = 16.66$) and CCI ($M = 28.64$, $SD = 16.38$) mice fell off the steady rotarod trial more quickly than naïve mice ($M = 43.41$, $SD = 13.73$) ($F(2, 45) = 4.46$, $p < .05$; Figure 24B). On the more challenging accelerating rotarod trial on day 2, CCI ($M = 17.87$, $SD = 4.95$) mice fell off more quickly than naïve ($M = 24.80$, $SD = 8.41$) ($F(2, 45) = 3.97$, $p < .05$; Figure 24D).
Figure 24. Motor deficits were evident on the rotarod. (A) Differences between naïve, craniotomy, and CCI mice were not detected on day 1 of the steady rotarod trial ($p > .05$). (B) Craniotomy ($p < .05$) and CCI ($p < .05$) mice fell off the rotarod on day 2 more quickly than naïve. (C) Differences between naïve, craniotomy, and CCI mice were not detected on day 1 of the accelerating rotarod trial ($p > .05$). (D) CCI ($p < .05$) mice fell off the accelerating rotarod on day 2 more quickly than naïve.

Similar results were observed on day 2 of the most challenging rotarod test. Craniotomy ($M = 15.30, SD = 6.86$) and CCI ($M = 13.56, SD = 4.03$) mice fell off the trial more quickly than naïve mice ($M = 19.99, SD = 5.64$) ($F(2, 45) = 5.06, p < .01$; Figure 25B).
Figure 25. Motor deficits were evident on the rotarod. (A) Differences between naïve, craniotomy, and CCI were not detected on day 1 of the faster rotarod trial ($p > .05$). (B) Craniotomy ($p < .05$) and CCI ($p < .05$) mice fell off the fast rotarod trial on day 2 more quickly than naïve.

Male and female mice did not differ in performance on the steady (Figure 26), accelerating (Figure 26), and faster (Figure 27) rotarod trials on day 1 and 2 ($p > .05$).
**Figure 26.** Motor performance was not influenced by gender. (A, B) Male and female mice did not differ in performance on the steady rotarod trials on day 1 and 2 ($p > .05$). (C, D) Male and female mice did not differ in performance on the accelerating rotarod trials on day 1 and 2 ($p > .05$).

**Figure 27.** Motor performance was not influenced by gender. (A, B) Male and female mice did not differ in performance on the faster rotarod trial on day 1 or 2 ($p > .05$).
General anxiety behaviors were assessed with the elevated zero maze. The performance of the maze did not differ between naïve, craniotomy, and CCI mice ($p > .05$; Figure 28). Similarly, gender did not affect the performance in the zero maze ($p > .05$; Figure 29).

**Figure 28.** Mice did not differ in anxiety-like behaviors. (A) Naïve, craniotomy, and CCI mice did not differ in the percentage of time spent in the dark in the zero maze on day 1 ($p > .05$). (B) Naïve, craniotomy, and CCI mice did not differ in the percentage of time spent in the dark in the zero maze on day 2 ($p > .05$). Overall, mice did not differ in the average percentage of time spent in the dark ($p > .05$).
Figure 29. Male and female mice did not differ in anxiety-like behaviors. (A) Mice did not differ in the percentage of time they spent in the dark on day 1 of the zero maze ($p > .05$). (B) Mice did not differ in the percentage of time they spent in the dark on day 2 of the zero maze ($p > .05$). (C) Overall, mice did not differ in the average percentage of time spent in the dark ($p > .05$).

Brain injury produced severe learning deficits in the cued water maze. Both CCI ($M = 645.87, SD = 142.83$) and craniotomy ($M = 498.43, SD = 220.57$) mice performed worse on the cued water maze by swimming longer distances compared to naïve ($M = 359.08, SD = 120.09$) ($F(2, 45) = 9.98, p < .01$; Figure 30A). CCI ($M = 34.53, SD = 10.00$) and craniotomy ($M = 34.53, SD = 10.00$) mice took longer to find the platform than naïve ($M = 18.99, SD = 6.80$) ($F(2, 45) = 9.94, p < .01$; Figure 30C), and CCI ($M = 10017.23, SD = 3605.78$) mice showed no evidence of learning the platform’s location.
compared to naïve \((M = 5572.91, SD = 2340.52)\) \((F(2, 45) = 7.18, p < .01; \text{Figure 30B})\). Injury severity did not affect the average distance to the platform \((p > .05; \text{Figure 30D})\).

*Figure 30.* Brain injury induced significant learning deficits in the cued water maze. (A) Both CCI \((p < .01)\) and craniotomy \((p < .05)\) mice searched the maze more than naïve mice. (B) CCI mice had a cumulative distance to the platform than naïve mice \((p < .01)\). (C) Both CCI \((p < .01)\) and craniotomy \((p < .05)\) mice took longer to find the platform than naïve mice. (D) Injury severity did not affect the average distance to the platform \((p > .05)\).

Gender did not affect performance in the cued water maze \((p > .05; \text{Figure 31})\).
Figure 31. Gender did not influence water maze performance. (A) Male and female mice did not differ in the distance traveled in the cued water maze ($p > .05$). (B) Male and female mice did not differ in the time it took to locate the platform in the cued water maze ($p > .05$). (C) Male and female mice did not differ in the cumulative swim distance to the platform in the cued water maze ($p > .05$). (D) Male and female mice did not differ in the averaged distance to the platform in the cued water maze ($p > .05$).

Learning and memory were assessed in the spatial water maze. Naïve, craniotomy, and CCI mice did not differ in the distance traveled in the spatial water maze ($p > .05$; Figure 32).
Distance traveled was not influenced by injury in the water maze. (A) Naïve, craniotomy, and CCI mice did not differ in the distance moved in the spatial 1 water maze ($p > .05$). (B) Naïve, craniotomy, and CCI mice did not differ in the distance moved in the spatial 2 water maze ($p > .05$). (C) Naïve, craniotomy, and CCI mice did not differ in the distance moved in the spatial 3 water maze ($p > .05$).

CCI mice ($M = 36.91, SD = 11.53$) performed worse compared to naïve ($M = 26.20, SD = 9.23$) when the platform’s location was changed on day 2 of the water maze, $F(2, 45) = 4.52, p < .05$; Figure 33A. These differences were minimized on day 3 of the water maze (Figure 33B), but returned on day 4 (Figure 32C). CCI mice ($M = 37.49, SD = 3.41$) performed worse compared to naïve ($M = 26.15, SD = 2.77$) when the platform was changed on day 4 of the water maze, $F(2, 45) = 482.38, p < .05$; Figure 33C.
Figure 33. Learning and memory deficits were evident in the water maze. (A) CCI mice took longer to locate the submerged platform than naïve mice on day 1 of the spatial water maze ($p < .05$). (B) Mice did not differ in the time it took to locate the platform on day 2 of the spatial 2 water maze ($p > .05$). (C) CCI mice took longer to locate the submerged platform than naïve mice on day 3 of the spatial water maze ($p < .05$).

CCI mice ($M = 9029.57$, $SD = 1053.06$) traveled a longer cumulative distance to the platform compared to naïve ($M = 6022.30$, $SD = 584.41$) on day 1 of the spatial water maze, $F(2, 45) = 4.15$, $p < .05$; Figure 34A. These differences were minimized on day 2 of the spatial water maze (Figure 34B), but returned in spatial 3 (Figure 34C) and probe trials (Figure 34C) of the water maze. CCI mice ($M = 11151.42$, $SD = 1476.66$) traveled a longer cumulative distance to the platform compared to naïve ($M = 6397.08$, $SD = 679.68$) in the spatial 3 water maze, $F(2, 45) = 5.07$, $p < .01$; Figure 34C. Similarly, CCI mice ($M = 45.83$, $SD = 1.13$) traveled a greater cumulative distance to the platform.
compared to naïve ($M = 39.45, SD = 1.35$) in the probe trials, $F(2, 45) = 5.03, p < .01$; Figure 34D.

Figure 34. Learning and memory deficits were evident in the water maze. (A) CCI mice swam longer distances to locate the submerged platform than naïve in the spatial 1 water maze ($p < .05$). (B) There were no differences in swim distance in the spatial 2 water maze ($p > .05$). (C) CCI mice swam longer distances to locate the platform than naïve in the spatial 3 water maze ($p < .01$). (D) CCI mice swam longer distances in search of the platform than naïve in the probe trials ($p < .01$).

CCI mice ($M = 36.34, SD = 1.78$) swam a longer distance to the platform than naïve ($M = 31.65, SD = 1.12$) in the spatial 1 water maze, $F(2, 45) = 3.51, p < .05$; Figure 35A. These differences were not found in the spatial 2 water maze (Figure 35B), but returned in the spatial 3 and probe trials. CCI mice ($M = 44.27, SD = 2.48$) swam a longer distance to the platform than naïve ($M = 36.57, SD = 0.83$) in the spatial 3 water maze, $F(2, 45) = 5.63, p$
Similarly, CCI mice ($M = -4.48, SD = 1.78$) swam longer distances to the platform than naïve ($M = -0.75, SD = 0.43$) in the probe trials, $F(2, 45) = 6.24, p < .01$; Figure 35D.

**Figure 35.** Learning and memory deficits were evident in the water maze. (A) CCI mice swam longer distances to find the platform than naïve in the spatial 1 water maze ($p < .05$). (B) There were no differences between injury groups in the spatial 2 water maze ($p > .05$). (C) CCI mice swam longer distances to find the platform than naïve in the spatial 3 water maze ($p < .01$). (D) CCI mice swam longer distances to find the platform than naïve in the probe trials of the water maze ($p < .01$).

Overall, CCI mice had a significant left sided turn bias in the water maze. CCI mice ($M = -39.08, SD = 15.77$) swam with a significant left sided turn bias compared to naïve ($M = 3.09, SD = 6.22$) and craniotomy ($M = -3.70, SD = 6.12$) in the spatial 1 water maze, $F(2, 45) = 5.65, p < .01$; Figure 36A). CCI mice ($M = -31.48, SD = 14.37$) swam with a
significant left sided turn bias compared to naïve ($M = -0.15, SD = 4.66$) in the spatial 2 water maze, $F(2, 45) = 3.84, p < .05$; Figure 36B). CCI mice ($M = -34.65, SD = 15.91$) swam continued to swim with a left sided turn bias in the spatial 3 water maze compared to naïve ($M = -2.25, SD = 6.12$), $F(2, 45) = 3.10, p < .05$; Figure 36C). CCI mice ($M = -39.74, SD = 16.42$) also swam with a left sided turn bias in the probe trials compared to naïve ($M = -2.42, SD = 5.50$) and craniotomy ($M = -7.56, SD = 5.21$), $F(2, 45) = 4.74, p < .01$; Figure 36D).

**Figure 36.** CCI mice swam with a left sided turn bias in the water maze. (A) CCI mice swam with a significant left sided turn bias compared to naïve ($p < .01$) and craniotomy ($p < .05$) in the spatial 1 water maze. (B) CCI mice also swam with a significant left sided turn bias compared to naïve in the spatial 2 water maze ($p < .05$). (C) CCI mice continued to swim with a left sided turn bias compared to naïve in the spatial 3 water maze ($p < .05$). (D) CCI mice swam with a significant left sided bias in all of the probe trials compared to naïve ($p < .01$) and craniotomy ($p < .05$).
Although CCI mice had severe motor deficits, swim speed did not differ in the spatial and probe water maze ($p > .05$; Figure 37).

Figure 37. Swim speed did not differ in the water maze. (A) Swim speed did not differ between groups in the spatial 1 water maze ($p > .05$). (B) Swim speed did not differ between groups in the spatial 2 water maze ($p > .05$). (C) Swim speed did not differ between groups in the spatial 3 water maze ($p > .05$). (D) Swim speed did not differ between groups in the probe trials of the water maze ($p > .05$).

Overall, gender did not affect performance in the spatial water maze ($p > .05$).

Male and female mice did not differ in the distance moved (Figure 38), time to find the submerged platform (Figure 39), cumulative distance swam (Figure 40), average distance to the platform (Figure 41), swim turn bias (Figure 42), and swim speed (Figure 43).
Figure 38. Gender did not influence swim distance in the water maze. (A) Male and female mice did not differ in the distance moved in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ the distance moved in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in the distance moved in the spatial 3 water maze ($p > .05$).
Figure 39. Gender did not influence time to the platform in the water maze. (A) Male and female mice did not differ in the time it took to find the submerged platform in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ in the time it took to find the submerged platform in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in the amount of time it took to find the submerged platform in the spatial 3 water maze ($p > .05$).
Figure 40. Gender did not influence the cumulative distance mice swam in the water maze. (A) Male and female mice did not differ in the total distance swam in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ in the total distance swam in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in the total distance swam in the spatial 3 water maze ($p > .05$). (D) Male and female mice did not differ in the total distance swam in the probe water maze trials ($p > .05$).
Figure 41. Gender did not influence the average distance to the platform in the water maze. (A) Male and female mice did not differ in the average distance swam to the platform in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ in the average distance swam to the platform in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in the average distance swam to the platform in the spatial 3 water maze ($p > .05$). (D) Male and female mice did not differ in the average distance swam to the platform in the probe water maze trials ($p > .05$).
Figure 42. Gender did not influence turn bias in the water maze. (A) Male and female mice did not differ in swim turn bias in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ in swim turn bias in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in swim turn bias in the spatial 3 water maze ($p > .05$). (D) Male and female mice did not differ in swim turn bias in the probe water maze trials ($p > .05$).
Figure 43. Gender did not influence swim speed in the water maze. (A) Male and female mice did not differ in swim speed in the spatial 1 water maze ($p > .05$). (B) Male and female mice did not differ in swim speed in the spatial 2 water maze ($p > .05$). (C) Male and female mice did not differ in swim speed in the spatial 3 water maze ($p > .05$). (D) Male and female mice did not differ in swim speed in the probe water maze trials ($p > .05$).

More detailed analysis of the probe trials revealed injury dependent deficits. CCI mice ($M = 29.32$, $SD = 1.87$) spent the least time searching for the platform in the area it was located the day before (target quadrant) than naïve ($M = 22.58$, $SD = 1.51$), $F(2, 45) = 4.96$, $p < .01$; Figure 44B). Diet (Figure 44A) and gender (Figure 44C) did not affect the time they spent searching for the platform in the correct quadrant ($p > .05$).
Figure 44. Injury severity influenced the time mice spent searching for the platform in the water maze. (A) The grape-enriched diet did not increase the amount of time mice spent searching for the platform in the location it was in the day before in the probe water maze trials ($p > .05$). (B) CCI mice spent the least time searching for the platform in the location it was in the day before compared to naïve in the probe water maze trials ($p < .01$). (C) Male and female mice did not differ in the time spent searching for the platform in the location it was in the day before in the probe water maze trials ($p > .05$).

Overall, there were no differences in the amount of time mice spent searching for the platform in the opposite location during the probe water maze trials (Figure 45; $p > .05$).
Figure 45. Mice did not differ in the time spent looking for the platform in the opposite location. (A) The grape-enriched diet did not reduce the amount of time mice spent looking for the platform in the opposite location (\( p > .05 \)). (B) Naïve, craniotomy, and CCI mice did not differ in the amount of time spent looking for the platform in the opposite location (\( p > .05 \)). (C) Male and female mice did not differ in the amount of time spent looking for the platform in the opposite location (\( p > .05 \)).

Similar to spatial water maze data, CCI mice (\( M = 45.83, SD = 1.13 \)) swam longer distances searching for the platform in the probe trials than naïve (\( M = 39.45, SD = 1.35 \)), \( F(2, 45) = 5.04, p < .01 \); Figure 46B). Diet (Figure 46A) and gender (46C) did not affect the total swim distance in the probe water maze trials (\( p > .05 \)).
Figure 46. Injury severity influenced the swim distance mice swam searching for the platform in the water maze. (A) The grape-enriched diet did not reduce the distance mice swam in search of the platform ($p > .05$). (B) CCI mice swam a longer distance in search of the platform compared to naïve in the probe water maze trials ($p < .01$). (C) Male and female mice did not differ in the total swim distance in the probe water maze trials ($p > .05$).

Most interestingly, the grape-enriched diet had a significant positive impact in craniotomy mice. Craniotomy mice ($M = 9011.33, SD = 749.79$) on the enriched diet swam shorter distances searching for the platform than control ($M = 12927.45, SD = 1426.49$) in the spatial 2 water maze, $F(1, 14) = 4.97, p < .05$; Figure 47A). Similarly, craniotomy mice ($M = 40.48, SD = 1.98$) on the enriched diet swam a shorter average distance to the platform than control ($M = 48.18, SD = 1.71$) in the spatial 2 water maze, $F(1, 14) = 8.73, p < .01$; Figure 47B). Lastly, male craniotomy mice ($M = 37.63, SD = 1.53$)
1.11) swam a shorter average distance to the platform than females ($M = 41.58$, $SD = 1.57$) in the spatial 3 water maze, $F(1, 46) = 4.20$, $p < .05$; Figure 47C).

**Figure 47.** Diet and gender had a significant effect in craniotomy mice. (A) Craniotomy mice on the enriched diet swam shorter distances in search of the submerged platform in the spatial 2 water maze ($p < .05$). (B) Craniotomy mice on the enriched diet also swam a shorter average to the platform in the spatial 2 water maze ($p < .01$). (C) Male craniotomy mice swam a shorter average distance to the platform in the spatial 3 water maze ($p < .05$).

Overall, injury severity did not affect depression-like behaviors (Figure 48).
Figure 48. Injury did not influence depressive-like behavior in the tail suspension test (A) Naïve, craniotomy, and CCI mice did not differ in the total time spent immobile ($p > .05$). (B) Naïve, craniotomy, and CCI mice did not differ in the time they first became immobile during the test ($p > .05$). (C) Naïve, craniotomy, and CCI mice did not differ in the time they spent immobile during the last four minutes of the test ($p > .05$). (D) Naïve, craniotomy, and CCI mice did not differ in the total percentage of time spent immobile ($p > .05$).

Similarly, differences between male and female mice were not detected in the tail suspension test (Figure 49).
Figure 49. Gender did not influence depressive-like behavior in the tail suspension test. (A) Male and female mice did not differ in the total amount of time spent immobile on the test ($p > .05$). (B) Male and female mice did not differ in the amount of time spent immobile during the last 4 minutes of the test ($p > .05$). (C) Male and female mice did not differ in the time they first became immobile in the test ($p > .05$).

Aim 3: The Influence of a Grape-Enriched Diet and/or Brain Injury on Alzheimer’s Disease Neuropathology

Influence of Diet on Alzheimer’s Disease Neuropathology

The grape-enriched diet did not influence total amyloid (Figure 50A) or fibrillar (Figure 50B) plaque burden in the brain.
Figure 50. Diet did not affect Alzheimer’s disease pathology. (A) The enriched diet did not reduce total plaque burden in the brain \((p > .05)\). (B) The enriched diet did not reduce fibrillar plaque burden in the brain \((p > .05)\).

**Influence of Injury on Alzheimer’s Disease Neuropathology**

Brain injury did not increase AD neuropathology. In fact, craniotomy mice \((M = .39, SD = .08)\) had lower total Aβ in the brain than naïve mice \((M = .68, SD = .08)\) \((F(2, 35) = 3.90, p < .05; \text{Figure 51A})\). Plaque load did not differ in CCI mice \((M = .11, SD = .05)\) compared to naïve and craniotomy mice \((p > .05)\). CCI mice less fibrillar Aβ in the brain compared to naïve \((M = .23, SD = .03)\) \((F(2, 33) = 3.72, p < .05; \text{Figure 51B})\).
Figure 51. Brain injury influenced AD neuropathology. (A) Craniotomy mice had lower Aβ plaque burden in the brain compared to naïve mice ($p < .05$). (B) CCI mice had lower fibrillar Aβ plaque burden in the brain compared to naïve mice ($p < .05$).

Representative pictures of total Aβ plaque and fibrillar Aβ plaque staining in the brain are shown in Figures 52 and 53, respectively.

Figure 52. Representative staining for total Aβ plaque staining in the brain. Bright spots are positive staining. (A) Higher amounts of positive staining were found in naïve mice. (B) Craniotomy mice had fewer amounts of positive staining. (C) CCI mice had the least amount of positive staining in the brain.
Figure 53. Representative staining for fibrillar Aβ plaque staining in the brain. Bright spots are positive staining. (A) Higher amounts of positive staining were found in naïve mice. (B) Craniotomy mice had fewer amounts of positive staining, but did not differ from naïve or CCI. (C) CCI mice had the least amount of positive staining.

**Interactions between the Enriched Diet and Injury**

Brain injured mice on the enriched diet did not have smaller lesions or Aβ deposition in the brain ($p > .05$; Figure 54).
Figure 54. Interactions between brain injury and diet were not found. (A) Craniotomy mice on the enriched diet had a reduced lesion volume compared to control ($p < .05$). Diet did not affect lesion volume in CCI mice ($p > .05$). (B) Injured mice on the grape-enriched diet did not have reduced $\alpha$B deposition in the brain ($p > .05$). (C) Injured mice on the grape-enriched diet did not have reduced fibrillar $\alpha$B deposition in the brain ($p > .05$).

Aim 4: The Influence of a Grape-Enriched Diet and/or Brain Injury on Blood Brain Barrier Clearance and Astrogliosis

Influence of Diet on BBB Clearance and Astrogliosis

The grape-enriched diet did not result in a reduction of RAGE (Figure 55A) and GFAP expression. In fact, mice on the enriched diet ($M = .06$, $SD = .004$) had more GFAP expression than control ($M = .05$, $SD = .003$) ($F(1, 39) = 4.46$, $p < .05$; Figure 55B).
Figure 55. The grape-enriched diet did not reduce RAGE or GFAP expression in the brain. (A) Diet did not influence RAGE expression in the brain ($p > .05$). (B) The grape-enriched diet led to increased GFAP expression in the brain ($p < .05$).

Influence of Injury on BBB Clearance and Astrogliosis

Brain injury did not affect RAGE or GFAP expression in the brain (Figure 56).

Figure 56. Brain injury did not influence BBB clearance or astrogliosis. (A) Brain injury did not affect the expression of RAGE in the brain ($p > .05$). (B) Brain injury did not affect the expression of GFAP in the brain ($p > .05$).

Interactions between the Enriched Diet and Injury

Brain injured mice on the enriched diet did not have less RAGE and astroglial activity (GFAP) in the cortex ($p > .05$; Figure 57).
Figure 57. Interactions between brain injury and diet were not found. (A) Brain injured mice on the enriched diet did not have reduced RAGE expression in the cortex ($p > .05$). (B) Brain injured mice on the enriched diet did not have reduced astroglial activity in the cortex ($p > .05$).
Correlations

**Grape-Enriched Diet**

Grape-enriched mice had improved in activity, but increased in affective behaviors across tests. Specifically, grape-enriched mice that had smaller lesions demonstrated better motor skills and coordination, \( r = -0.60, p = 0.039 \) (Figure 58).

Furthermore, grape-enriched mice that had smaller lesions had greater Aβ \( (r = -0.96, p = 0.00; \text{Figure 59}) \) and fibrillar Aβ deposition in the brain \( (r = -0.67, p = 0.048; \text{Figure 60}) \).

*Figure 58.* Grape-enriched mice with smaller lesions took longer to fall off the rotarod, \( p = 0.039 \).
Figure 59. Grape-enriched mice with smaller lesions had more Aβ deposition (HJ3.4) in the hippocampus, $p = .003$. 

\[ r = -.96 \]

\[ p < .01 \]
Figure 60. Grape-enriched mice with smaller lesions had more fibrillar Aβ deposition (ThioS) in the brain, $p = .003$.

Grape-enriched mice that displayed less avoidance/anxiety behavior on the zero maze (Day 1), took longer to find the platform in the Spatial 1 water maze ($r = -.45, p = .029$; Figure 61). Similarly, grape-enriched mice that displayed less avoidance/anxiety behavior on the zero maze (Day 2), took longer to give up (i.e. less depressed) on the tail suspension test ($r = -.41, p = .046$; Figure 62). Interestingly, grape-enriched mice that displayed less depressive-like behavior on the tail suspension test had more fibrillar Aβ deposition in the cortex ($r = -.69, p = .002$; Figure 63).
Figure 61. Grape-enriched mice that spent less time in the dark in the zero maze took longer to find the submerged platform in the Spatial 1 water maze, $p = .029$. 
Figure 62. Grape-enriched mice that spent less time in the dark in the zero maze spent more time struggling before becoming immobile in the tail suspension test, $p = .046$. 

\[ r = -.41^* \]

\[ p < .05 \]
Figure 63. Grape-enriched mice that spent the least time immobile had higher fibrillar Aβ deposition in the cortex, $p = .002$.

**Control Diet**

Whereas grape supplementation was associated with lesion size, plaque deposition, and affective behaviors, the control diet influenced motor skills and exploratory patterns in mice. Control mice with larger lesions took longer to find the platform in the Spatial 3 water maze ($r = .70, p = .008$; Figure 64).
Figure 64. Control mice with larger lesions spent more time to locate the submerged platform in the Spatial 3 water maze, \( p = .008 \).

Control mice that demonstrated better motor skills/coordination had reduced exploratory activity in the open field test \( (r = .44, p = .039; \text{Figure 65}) \). Surprisingly, mice that displayed less depressive-like behavior were also less efficient in the water maze by searching for the platform in the opposite quadrant in the Probe trials \( (r = -.56, p = .007; \text{Figure 66}) \).
Figure 65. Control mice that performed better on the rotarod also spent more time in the corner of the bin (did not explore) in open field testing, $p = .039$. 

$r = .44^*$

$p < .05$
Control mice that spent less time immobile also spent more time searching for the platform in the opposite quadrant in the Probe water maze trials, $p = .007$.

In addition, control mice that had reduced exploratory activity in the center of the open field test also took longer to locate the platform in the Spatial 1 water maze ($r = -.46, p = .048$; Figure 67). Similarly, control mice that did not explore the open field test also had increased Aβ deposition in the hippocampus ($r = .51, p = .053$; Figure 68).
Control mice that spent less time exploring in the open field test also took longer to find the submerged platform in the Spatial 1 water maze, $p = .029$. 

Figure 67. Control mice that spent less time exploring in the open field test also took longer to find the submerged platform in the Spatial 1 water maze, $p = .029$. 

$r = -.47$

$p < .05$
Figure 68. Control mice that spent more time in the perimeter of the open field test also had more Aβ deposition in the hippocampus, $p = .053$.

**Controlled Cortical Impacted Mice**

CCI mice were hyperactive and water maze performance was influenced by RAGE and reactive astrogliosis. For example, larger lesions were associated with hyperactive exploratory behaviors (increase in distance traveled; $r = .68, p = .03$; Figure 69). Hyperactive behaviors led to better search strategies in the Probe water maze trials, with CCI mice spending more time in the correct quadrant searching for the platform ($r = .73, p = .01$; Figure 70). Interestingly, slower exploration/activity was related to higher levels of RAGE in the cortex ($r = -.66, p = .04$; Figure 71) in CCI mice.
Figure 69. CCI that had larger lesions traveled farther in open field test, $p = .03$.
Figure 70. The faster CCI mice explored in open field test, the more time they spent searching for the submerged platform in the correct quadrant in Probe water maze trials, \( p = .01 \).
CCI mice that had more astrogliaosis did not display depressive-like behaviors (longer to become immobile in the tail suspension test; \( r = .61, p = .049 \); Figure 72), suggesting that these mice may have had activated repair mechanisms to reduce apoptosis in the cortex. Similarly, CCI mice with reduced RAGE expression performed better in the Probe water maze trials \( (r = -.80, p = .006; \) Figure 73), by spending more time searching for the platform in the correct quadrant. Lastly, increased RAGE expression led to more fibrillar Aβ deposition in the cortex \( (r = .80, p = .029; \) Figure 74).
Figure 72. CCI mice that had more astrogliosis in the cortex also took longer to become immobile in the tail suspension test, $p = .049$. 

$r = .61^{*}$  
$p < .05$
Figure 73. CCI that had reduced RAGE expression in the cortex spent more time searching for the platform in the correct quadrant in Probe water maze trials, $p = .006$. 

$r = -.80$

$p < .01$
Figure 74. CCI mice that had increased RAGE expression in the cortex also had increased fibrillar Aβ deposition in the brain, $p = .029$. 
CHAPTER FOUR

DISCUSSION

Animal models have essential to understanding the behavioral and biological consequences of AD. These models have shown the development and progression of the disease similar to those reported in clinical populations. The purpose of this study was to explore the effects of TBI on behavior and plaque burden in a mouse model of AD, as well as the protective effects of a grape-enriched diet in AD and TBI. We found the grape-enriched diet to be more protective in mice with mild brain injury (craniotomy) than those with moderate to severe brain injury. Our findings also show that brain injury may have protected against Aβ pathology in the brain.

Effects of Diet, Injury, and Gender on Behavior

CCI is a model commonly used to model the pathological and behavioral features of moderate to severe injury in the human TBI population. We have shown that mild CCI in rodents causes extensive white matter tissue disruption (Donovan et al., 2012; Donovan et al., 2014) and moderate CCI leads to motor and behavioral impairments (Ajao et al., 2012; Brody et al., 2007; Kamper et al., 2013), confirming our current findings. The moderate nature of the injury produced more tissue damage and motor deficits than the craniotomy mice. General exploratory activity levels are also altered in CCI mice and rats, with increased anxious activities and less likely to engage in exploratory behavior, though hyperactivity has also been reported. Similar to our correlative results, TBI rodents have been reported to display hyperactivity in open field testing (Hsieh et al., 2014; Li et al., 2006). In addition, several studies have reported
behavioral impairments are present immediately following TBI (Ajao et al., 2012; Hartman et al., 2012; Kamper et al., 2013, confirming our results in the rotarod and performance in the standard water maze and relative angular velocity (turn bias). CCI mice had impaired performance in the water maze, taking longer to learn the location of the platform each day. These mice also showed significant impairment in the probe trials. Surprisingly, mild-injured craniotomy mice also displayed motor deficits on the rotarod and learning deficits during the cued learning trials of the water maze, though other studies have reported impairments found in larger mild injuries (Donovan et al., 2012, Donovan et al., 2014; Washington et al., 2012).

Many studies have shown polyphenols to be neuroprotective and supplementation reduces inflammation (Ates et al., 2007; Gomez-Serranillos et al., 2009; Loren et al., 2005; Wang et al., 2009) and AD neuropathology (Hartman et al., 2006; Wang et al., 2006; Ho et al., 2009). Our study reports that the grape-enriched diet produced smaller brain lesions, fewer sensorimotor deficits in craniotomy mice, though results did not reach statistical significance. Perhaps most surprising, the grape-enriched diet was found to be the most influential in mild injured craniotomy mice only compared to CCI. Interestingly, the craniotomy surgery seemed to produce brain damage that mimicked mild brain injury and its corresponding behavioral deficits. These results indicate that the grape diet may have a small, but positive effect on motor activity in mild brain injury. Interestingly, the polyphenol diet significantly influenced learning and memory in craniotomy mice, consistent with several studies (Dulcich et al., 2013; Hartman et al., 2006). In addition grape-enriched craniotomy male mice found the location of the platform faster than females. Females did not dominate performance over males.
previously published (Dulcich et al., 2013), though the injury mechanism and polyphenol supplementation were different.

Some studies report that polyphenols produce antidepressant-like activity similar to the effects of pharmaceuticals (Liu et al., 2013; Wang et al., 2013; Zhu et al., 2012). In contrast, our study found that the grape-enriched diet resulted in more depressive-type of behaviors in CCI mice compared to controls. Our findings may be attributed to the specific interactions between grape polyphenols, TBI and AD neuropathology. The AD neuropathology in these mice may mask polyphenolic properties that have shown positive results in other studies. Further studies should investigate the mechanistic pathways polyphenols affect the brain.

Effects of Diet and Injury on AD neuropathology and the BBB

Polyphenol supplementation has also been shown to reduce AD neuropathology (Hartman et al. 2006; Ho et al., 2009; Joseph et al., 2003; Wang et al., 2006). Many studies have reported that TBI exacerbates AD neuropathology exponentially (Gentleman et al., 1997; Roberts et al., 1994; Uryu et al., 2002; Valle et al., 2001), however, we found that brain injury did not up-regulate Aβ mediating BBB proteins. In fact, we report that TBI, not the grape-enriched diet, significantly reduced AD neuropathology. Because the grape diet was started at around the age when Aβ plaques are just beginning to appear in PSAPP mice and may have influenced the time course of Aβ aggregation and accumulation in the brain. Naïve mice had more Aβ and fibrillar Aβ compared to craniotomy and CCI mice, respectively. Although this finding is controversial, some studies have found that TBI reduces AD plaque burden in the brain. Aβ plaques have
been found shortly after TBI in adults, however few plaques were found several months to years despite accumulation of Aβ in the axons (Chen, Johnson, Uryu, Trojanowski & Smith 2008). Hippocampal plaque burden is also found to regress in the ipsilateral hippocampus several weeks post-TBI, compared to the contralateral hippocampus (Nakagawa et al., 2000). In fact, the loss of ipsilateral hippocampal CA3 neurons by CCI may be responsible for the short-term increase in Aβ levels (Smith et al., 1998), whereas the over expression of Bcl-2 (B-Cell CLL/Lymphoma 2) post-injury may be responsible for the long-term neuroprotection (Nakamura et al., 1999). The Bcl-2 gene is known to inhibit both apoptosis and necrosis cell death initiated by brain injury. Alternatively, the acute increase in Aβ caused by the initial injury may not compromise neurons enough to engage in long-term amyloid accumulation. Additional injuries may be necessary for neurons to become more susceptible and process higher concentrations of Aβ, leading to more degeneration over time (Nakagawa et al., 1999).

Grapes have also been found to protect against oxidative damage and promote astrogliosis. Cell culture treated with GSE increased hippocampal astrocyte activity against oxidative stress (Fujishita et al., 2009). Increased astrocyte activity was also found in cells that were pre-treated with wine (Gomez-Serranillos et al., 2009). This is similar to what we demonstrated in this study. We found that grape-enriched mice had increased astroglial activity in the cortex, suggesting the significant role polyphenols play in mediating oxidative activity.

**Future Directions**

Determining the mechanisms that TBI reduces Aβ pathogenesis is beyond the
scope of the current study. Future projects should focus on determining the levels of oxidative stress, apoptosis, or Bc1-2 to better understand the attenuation mechanisms of TBI in AD. Also, biochemistry (Elisa kits) of fresh frozen tissue would confirm whether the controversial findings from this study are indeed true. Further, swim strategy analysis may also provide a more sensitive detection of whether grape-enriched mice used different search techniques to find the platform.

**Conclusions**

Our findings suggest that TBI reduced the production of Aβ. Instead behavioral deficits may have been a result of injury severity, rather than AD pathology. Injury induced motor and behavioral deficits were prominent, consistent to literature. Surprisingly, the grape-enriched diet was far more protective in a mild brain injury and more detrimental in moderate injury.
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