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Alzheimer's Disease and Diabetes: A Transgenic Mouse Model in Behavior, MRI, and Cells

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Doctor of Philosophy

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ABSTRACT

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Alzheimer's disease (AD) is the most common form of dementia, afflicting almost 5 million patients in the US, and impacting millions more, financially, physically and emotionally. Coming in as the 6th leading cause of death in the US, and showing no signs of slowing its annually increasing rates, the world is in desperate need of improved understanding of the disease's multifaceted pathogenesis and progression, more accurate forms of detection and diagnosis, and more effective prevention and treatment.

While many are focused on the noble pursuit of understanding the genetic contributions to the appearance of the pathological amyloid beta (Aβ) plaques and tau tangles seen in AD, the majority of cases are not explained by genes or allele risk. Instead environmental, dietary and lifestyle contributors may be the key to understanding, diagnosing and treating this awful disease. Diet especially may impact the body's ability to regulate oxidative stress, which will cause damage within the cell and lead to further dysregulation of iron storage and metabolism. Iron storage is heavily monitored through cellular mechanisms, and the way in which the body reacts involves creation of the Aβ plaques and tau tangles as receptacles for the molecule it has deemed as the cause of the problem, iron. We have aptly named our theory, the Iron Hypothesis, and in the following document will outline the evidence for this hypothesis, and the experiments designed and performed to prove it.

First, we aimed to examine the impacts that various treatments would have on a transgenic in-vivo model, examining the cohorts' behavior over several time points. We report a significant difference in the behavioral measures of time, distance, errors and failed trials in the radial arm maze existed between genotype, treatment and sex of the mice. Tissue of the experimental mice was collected, but will be processed and analyzed at a later date.

Secondly, we aimed to examine the same cohorts of the in-vivo mouse model for minute anatomical changes that took place over the course of the aforementioned behavioral trials using novel MRI scanning sequences sensitive to the low levels of iron build up. We report significant differences in the UTE scan measures for our western diet treatment at TE's of 1.2ms. Additionally, further investigation and optimization of the protocol may be required to further expand the findings.

Keywords: Alzheimer's disease, diabetes, MRI, amyloid beta, iron hypothesis, oxidative stress, homocysteine, tau

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CHAPTER 1: Introduction

With the world population reaching upwards of 7.6 billion (United Nations, 2017) there is an increasing concern for the worlds health. While the health of each person and age group is extremely important, advances in science and healthcare have allowed for a rapidly growing elderly population; a population that is especially at risk for developing dementia. A report published by the US Census Bureau projected an increase in the 65 and over population to reach 1.6 billion by 2050, which will mean a doubling of the aged while total population will only increase by 34 percent (He, Goodkind, & Kowal, 2016). Now if we apply that same doubling to the US, which is projected, the US 65 and over population is likely to reach nearly 100 million during the same period (Ortman, Velkoff, & Hogan, 2014). Projections from 2016 to 2050 published by the Alzheimer's Association put the US Alzheimer's patient population reaching 13.8 million and costing the country over 1 trillion dollars each year ("2016 Alzheimer's disease facts and figures," 2016). This devastating news reveals how great the need is for funding, public interest, and researchers devoted to Alzheimer's disease. The focus of my dissertation is centered on the need for developing a deeper understanding of Alzheimer's disease (AD), how certain aspects of the disease progress, identifying that progression earlier, and treating the disease before the harmful effects set in.

1.1 The Disease

Nearly everyone either knows or has heard of someone with Alzheimer's disease and can name the telltale signs of memory loss, confusion and irritability. Fewer people, however, realize that Alzheimer's disease is not simply a normal part of aging or that it has risen to become the 6th

leading cause of death in the US (National Center for Health, 2017). Traditionally, the cause of death is pinned on two proteins called amyloid beta (Aβ) and tau, both of which are found deposited in higher quantities in the brains of those with Alzheimer's disease. The aggregation of the aberrant proteins disrupts the function of the neurons in the brain, eventually killing them or forcing them into apoptosis. As an Alzheimer's patient loses an increasing number of neurons, they will begin to lose certain functions of their brain, typically beginning with memory. This loss of neurons and function continues until vital functions such as breathing and swallowing are lost and the person will pass (Neugroschl & Wang, 2011). Researchers have discovered many of the nuances to the different variations of AD but still cannot describe its genesis in many of the cases, but perhaps if we examine the history of human evolution we can find some clues about aging that will help us better understand AD.

1.2 The History of Aging

The few clues we have from the history of human evolution are found in the fossil record. Looking past its own inherent sampling biases, the fossil record holds subtle clues about the age and life expectancy of early humans, as well as some surprising similarities to modern humans. The first clue to our development is that old age only became more common later in the evolution of humans (Caspari & Lee, 2004). Old age helped the community to become more successful at surviving, which makes sense, as that community would have a greater collective experience. On average, however, the life expectancy of early humans was still somewhere between 20-40 years for hundreds of thousands of years, as evidenced by a much larger number of fossils that fall in that age group (Trinkaus, 2011). This, however, does not mean that people were incapable of living past the age of 40. In fact, many of the great philosophers and rulers of ancient civilizations are approximated to have lived well past their 60's (Aristotle at 62 and Genghis Khan at 65) and 70's

(Socrates and Charlemagne at 71) and, some even to their 80's (Plato at 82 and Ptolemy I Soter at 84). Despite the fact that it was possible to survive into old age, the average person alive before the 1900s did not. When Dr. Alois Alzheimer first characterized the disease in 1907, not as Alzheimer's disease but as a "peculiar disease"(Stelzma, 1995), the 65+ population had just barely begun to grow into significance, helping to raise the average age of humans. Since the turn of the century, that average has continued to improve, especially within the 0-4 years population, seeing a decrease in infant mortality by more than 90% between 1900-1997 (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). Lower infant mortality and a growing 65+ population have combined to dramatically improve the average age of the population. Simply put, more people are living longer than ever before, and it is becoming more and more apparent that the body and the brain are having a hard time keeping up with the changing times. It is not their fault either; with rapidly changing lifestyles and diets, the hardwired genetics are getting in the way of the increasing age.

1.3 The Genetics

One of the first things that receives blame with a disease like AD is faulty genetics, and while genetics are definitely a significant contributor to the millions of cases, it still does not account for the majority of the cases. Some of this may be simply due to the youth of the field of genetics, and that once more AD associated genes have been discovered, we will get a better handle on the disease itself. There have been major advances in identifying genes associated with development of AD, which typically splits the disease into two categories or forms of AD, that is: early-onset AD (EOAD) and late-onset AD (LOAD) (Ridge, Ebbert, & Kauwe, 2013). The two forms have unique genetic factors associated with them, and contribute to vastly different numbers of cases, yet they carry with them the same, or strikingly similar pathologies and outcomes.

1.3.1 Early Onset

Early-onset AD or "familial AD" is distinct from its sister form of the disease by its target age group. Age of onset is typically well before 65 years, ranging from 30 to 60 or 65 years (Bekris, Yu, Bird, & Tsuang, 2010). Unlike LOAD, the causative genetic factors account for nearly all the cases, ranging from an estimated 92 to 100% (Cacace, Sleegers, & Van Broeckhoven, 2016; Wingo, Lah, Levey, & Cutler, 2012). Additionally, EOAD has a prevalence of only 1-10% of all the cases of AD, depending on the study that is examined (Cacace et al., 2016; Campion et al., 1999; Ferri et al., 2005). The genetic contributions to EOAD have been identified as mutations to certain key genes that impact the production of proteins and enzymes associated with the disease, namely: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2). There are a variety of mutations that impact those genes, and nearly all the mutations lead to the development of AD. Even with the characterization of the genes as causative factors of the disease, their pathogenesis has still not been entirely elucidated, and there is sometimes intense debate concerning the pathway and interactions of the genes. Xia et. al. have shown that many of the mutations of the PSEN1 lead to an overall decrease in the Aβ production, going against the accepted amyloidosis hypothesis (Xia, Kelleher, & Shen, 2016; Xia et al., 2015). A counter paper by Veugelen et. al. contended that the original hypothesis holds and that while in some cases the overall Aβ production is decreased, the aberrant proteins that are produced would be better categorized as "seed" proteins, which then cause the massive plaque production cascade (Veugelen, Saito, Saido, Chavez-Gutierrez, & De Strooper, 2016). These varying explanations make it obvious that there is still a lack in understanding of the genetics involved.

1.3.2 Down Syndrome

Down syndrome (DS) is one of the few pre-existing conditions correlated with an increased rate of Alzheimer's disease besides just age. This increased rate is not seen in other intellectual disabilities, and therefore gives rise to the most accepted theory called the amyloid cascade theory. The theory postulates that the likely cause for the increased rates of AD in people with DS is due to the gene for APP, the parent protein of Aβ, being found on chromosome 21. Therefore, those with DS get an extra copy of APP, and along with it, an extra risk for Alzheimer's disease. Just as in other Alzheimer's patients, DS patients will develop Aβ plaques and tau tangles, but unlike the general population nearly 100 percent of people with DS will develop the typical AD pathologies. Interestingly, not all people with DS, despite having Aβ plaques and tau tangles, will develop the behavioral and clinical symptoms of AD. It is also interesting that the indicator of memory loss is not always the earliest reported symptom of those with DS, but instead myoclonic or generalized seizures (Castro, Zaman, & Holland, 2017). The reason for the difference in pathology and symptoms has yet to be elucidated, but could very possibly help to explain the relationship and interplay between genetics and environment in development of Alzheimer's disease.

1.3.3 Late Onset

The vast majority of AD cases, however, do not neatly fit into one or another of the many genetic boxes, which leads researchers to suspect the involvement of environmental contributions leading to "sporadic" AD. Sporadic, or late onset AD, is defined as occurring (being diagnosed) later than 60 or 65 years of age. LOAD has a prevalence close to 90% of all AD cases, and although a genetic component in LOAD is supported by twin studies, no consistent causative gene for the majority of cases has been identified. The only gene that is consistently associated with LOAD is the apolipoprotein E (APOE) gene (Bekris et al., 2010; Liu, Liu, Kanekiyo, Xu, & Bu, 2013). The

APOE gene, which encodes for apolipoprotein E (ApoE), has the function of clearing oligomerized amyloid β (oAβ) proteins from the brain by co-localizing with oAβ proteins after being secreted into the perivascular space (Rolyan et al., 2011), thereby resulting in a decrease in AD symptoms (Espiritu & Mazzone, 2008; Shea, Rogers, Ashline, Ortiz, & Sheu, 2002). Without fully functional ApoE molecules, oAβ proteins can accumulate in the extracellular space, forming plaques, facilitating the onset of AD. The functionality of ApoE in the body is determined by the allele combination present, and with a variety of allele combinations, there is varying risk of developing AD associated with each specific combination.

1.3.4 Apolipoprotein E Alleles

There are three different alleles for APOE: ε2, ε3, and ε4 – each of which codes for a protein which differs at only one or two amino acids at either position 112, 158 or both (Ghebranious, Ivacic, Mallum, & Dokken, 2005). Consequently, there are six different possible genotypes of APOE: ε2/2, ε2/3, ε2/4, ε3/3, ε3/4, and ε4/4. The ε2/2 genotype has the most reduced risk for AD, due to the protective effects allele ε2 has against AD (Corder et al., 1994). The ε2/3 genotype also carries a reduced risk for AD, while the ε2/4 and ε3/3 genotypes have a neutral contribution of risk for AD. Lastly the ε3/4 genotype has an elevated risk for AD, while the ε4/4 genotype has the greatest risk for AD (Castellano et al., 2011; Tsuang et al., 2013). The worldwide frequencies for the polymorphic alleles, ε2, ε3 and ε4 are 8.4%, 77.9% and 13.7 % respectively, however among AD patients the frequency of ε4 increases dramatically to ~40% (Ghebranious et al., 2005; Liu et al., 2013). Yet, even with this knowledge of the risk contribution of the different genes associated with AD, there are no current effective treatments, and further, the majority of LOAD cases do not fit into these genetic parameters. This suggests that the majority of cases are

a more complex mixture involving multiple, yet to be discovered, susceptibility genes in combination with a variety of environmental dietary or lifestyle factors (Bekris et al., 2010).

1.4 The Other Environmental Causes

We live in an increasingly industrial world, and because of this, we must consider the impacts in which that industrialization may have on our health. The rising populations of the world have created a need for larger amounts of food at a faster rate, and in order to accomplish this we have introduced things like pesticides, preservatives and automated manufacturing. There is a multitude of research examining the influence that various metals, pesticides and pollution have on the development of AD and related diseases (Dosunmu, Wu, Basha, & Zawia, 2007). Connect the rise of toxicological insults exposed to the average body, and specifically the nervous system, with the rapid rise in average lifespan increasing the time spent being exposed to those insults and the picture appears clear as to how AD rates are increasing at a commensurate rate. Just as prevalence and exposure to toxic chemicals in the environment has increased dramatically in recent human history, the average diet has been altered just as drastically. This change occurred in part due to necessity, but also out of ease, convenience and simple laziness. This has been evidenced by the increase in type 2 diabetes mellitus, which has been linked to obesity and a sedentary lifestyle (Hu, 2003). The Egyptians first described the symptoms of diabetes mellitus (DM) but it was not until the 17th or 18th century that physicians began to understand the disease well enough to treat it. They didn't have the modern medicines we have today, so they treated it with a change of diet and increase in exercise (Ahmed, 2002). While DM is not typically regarded as affecting the central nervous system, there are striking similarities that can be found between DM and AD as well as several studies that draw significant correlations between the two diseases.

1.4.1 Type 3 Diabetes

The first major study that showed a connection between AD and DM was called the "Rotterdam Study." The Rotterdam study began in 1990 with nearly 8000 participants age 55 and older, aimed at examining a variety of systems and potential diseases in the elderly (Hofman et al., 2009). Using data collected from 1990-1999 a group of researchers saw a near doubling of AD incidence in those previously diagnosed with DM. This incidence was doubled again within the group of diabetic patients who treated their condition with insulin (Ott et al., 1999). This correlation between the two diseases has lead researchers to unofficially name AD, "type 3 diabetes" (Ahmed, Mahmood, & Zahid, 2015). Unfortunately, the similarities do not stop there as a wealth of research has been published connecting the pathogenesis of several AD pathways to insulin function and regulation. De la Monte et al. showed a common finding of impairment in energy metabolism and glucose utilization, possibly stemming from an 80% reduction of insulin receptors, while Steen et al. showed a deficiency in insulin receptors, insulin itself and insulin-like growth factor, both of which further implicate insulin resistance in AD neuropathology (de la Monte & Wands, 2005; Steen et al., 2005). With insulin resistance implicated in AD pathogenesis one overarching commonality between DM and AD seems to rise to the top, and that is oxidative stress.

1.4.2 Oxidative Stress

Oxidation and reduction (redox) reactions are a part of the backbone chemical reactions that are absolutely necessary to sustain life. Without redox reactions, we lose our ability to metabolize nutrients, create energy, utilize oxygen, and maintain many other life-sustaining processes. A natural consequence of these redox reactions is the creation of free radicals and reactive oxygen species (ROS), which are harmful to our body and the building blocks out of

which our body is made. The damage that occurs as a result of these reactive molecules is called oxidative stress, but that is not the end of the story. Our body is equipped with mechanisms and signaling pathways to create antioxidants, molecules designed to combat free radicals and ROS, and reduce the overall oxidative stress in the cellular environment. What, however, happens when these safety systems are subverted, or diminished because of our body's exposure and reaction to the environment, diet and lifestyle that we subject it to? During pathological conditions, the redox balance within the cell is altered, producing toxic levels of uncontrolled ROS, which will cause damage to neurons. The oxidative damage seen in AD includes, but is not limited to, advanced glycation products (AGE) (Niwa et al., 1996; Zhou et al., 1998), nitration (Good, Werner, Hsu, Olanow, & Perl, 1996) and lipid peroxidation (Sultana, Perluigi, & Butterfield, 2013). There has been intense debate as to whether Aβ plaques and NFTs are a major source of oxidative free radicals promoting neurodegeneration, or whether they serve to compensate for or reduce oxidative damage (Hensley et al., 1994). This hypothesis of Aβ as an antioxidant is supported by observations of patients with Down's syndrome, where Aβ deposits are developed in late teens in response to increased oxidative stress (Cenini et al., 2012). There seems to be equal strength in either argument, denoting Aβ plaques and NFTs as precursors of oxidative damage or as responses to such insults, but no general consensus. Additionally, many observations provide proof that metals, such as iron (Hare, Ayton, Bush, & Lei, 2013b), aluminum (Crapper, Quittkat, Krishnan, Dalton, & De Boni, 1980), mercury (Xu et al., 2012), copper (Brewer, 2014) and zinc (Deibel, Ehmann, & Markesbery, 1996) all play a major catalytic role in production of free radicals and oxidative stress. As one of the most abundant in the body, iron has garnered the recent scientific limelight.

1.4.3 Iron and the Iron Hypothesis

Iron is an essential micronutrient whose metabolism is tightly regulated within the body and its normal and diseased physiological pathways are shown in Figure 1.1. Exchange and transport of iron is mediated by the protein, transferrin (Tf)(Anderson et al., 1987). Ferrous ions (Fe2+) are oxidized by ferroxidase to ferric ions (Fe3+) before being exported out of cell into the interstitium via ferroportin (FPN), a transmembrane protein (Aisen, Enns, & Wessling-Resnick, 2001). These Fe^{3+} ions are incorporated into Tf and are circulated via the blood (Finch & Huebers, 1982). The blood brain barrier prevents such complexes from diffusing into the nervous membrane; however, there seems to be another mechanism of endocytosis in the brain capillary endothelial cells (Hare, Ayton, Bush, & Lei, 2013a; Moos, Rosengren Nielsen, Skjorringe, & Morgan, 2007). The Tf-Fe^{3+} complexes are recognized by Tf receptors and are internalized in the cell through endosomes. The low pH inside the endosome converts Fe^{3+} to Fe^{2+} ions, which are then expelled out into the cytosol through divalent metal transporter 1 (Hentze, Muckenthaler, & Andrews, 2004). These free iron molecules in the cytosol constitute what is known as the labile iron pool (LIP), and are used for normal physiological functions. Iron can also be stored in a storage protein called ferritin (F) (Theil, 2004). Any excess iron is exported out to the interstitium by the only known iron exporting protein, FPN (Ganz, 2005), which is associated with a ferroxidase, oxidizing the iron ions (Abboud & Haile, 2000). The exact mechanism of ferroxidase activity in the brain is not entirely understood; however, ceruloplasmin (Cp), a potent ferroxidase, has been identified in its membrane anchored form in astrocytes (Patel, Dunn, & David, 2000; Patel et al., 2002). Researchers have yet to find a traditional ferroxidase enzyme active in neurons; however, APP has been shown to substitute Cp's function in regulating iron levels in neocortical neurons (Duce et al., 2010). With the oxidation state under control, iron is shuttled between ferritin and the LIP in order to maintain a constant level for cellular functions. The ebb and flow of iron

levels in the LIP are regulated by iron response proteins (IRP 1 & IRP 2), which are peptides that bind with iron response elements (IRE) at the ribosomal binding sites (RBS) (Aisen et al., 2001) of APP, ferritin and ferroportin, controlling their expression (Rogers et al., 2002). Disruptions in these regulations are found to promote neurodegeneration illnesses like AD (Antharam et al., 2012;

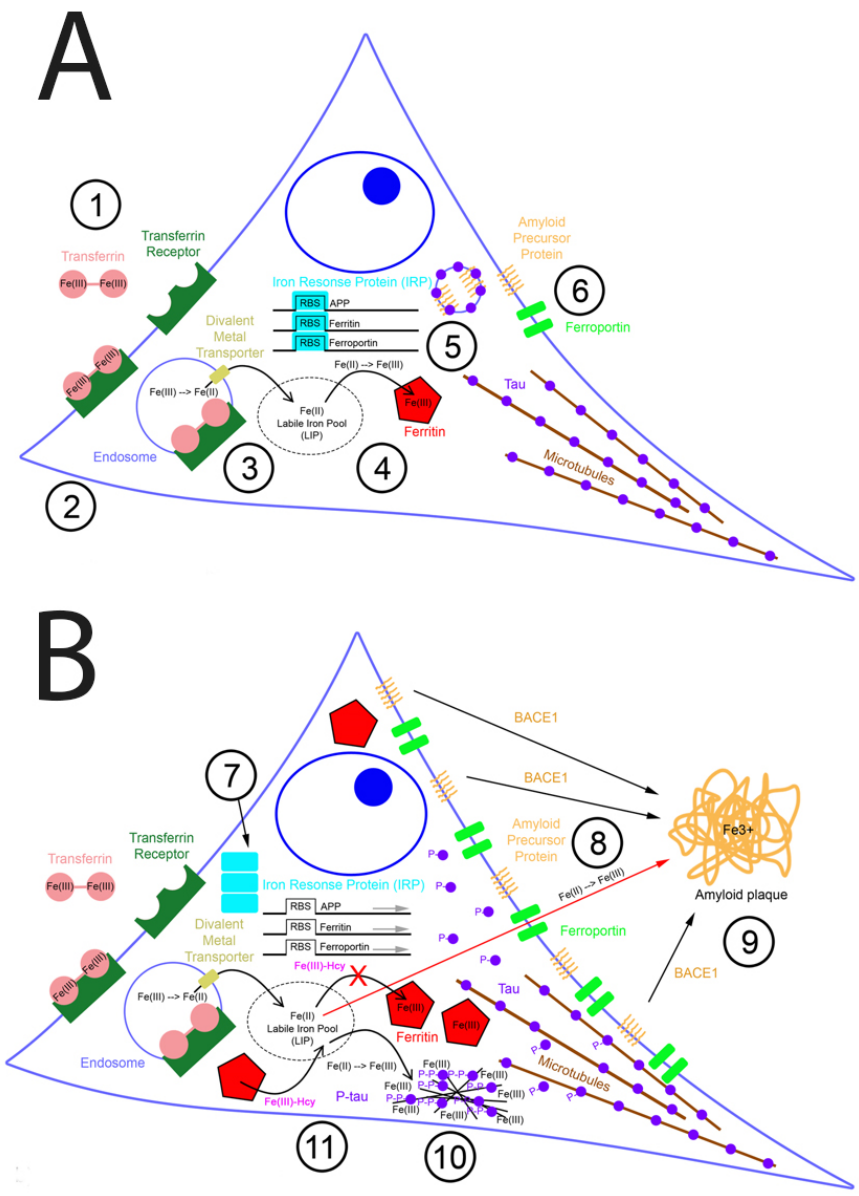

Figure 1.1: Iron Hypothesis. Iron storage, regulation and transport during (a) normal physiological conditions and (b) oxidative stress conditions in the diseased state.

Qin et al., 2011), and are associated with increased amyloid plaques and neurofibrillary tangles (Connor, Menzies, St Martin, & Mufson, 1992; Smith, Harris, Sayre, & Perry, 1997). Studies have demonstrated that disruptions in iron storage regulations cause neurons to develop high levels of iron in their redox active state (Good et al., 1996; Smith, Harris, Sayre, & Perry, 1997) prior to formation of Aβ plaques (Leskovjan et al., 2011), which were prevented by iron chelation therapy, suggesting a potential mechanism to target for treatment of iron dysregulation and potentially AD as well (Guo et al., 2013). Additionally, immunohistochemistry has revealed co-localization of iron with Aβ plaques and hyper-phosphorylated tau proteins (Mondragon-Rodriguez et al., 2013; Stamer, Vogel, Thies, Mandelkow, & Mandelkow, 2002). Such co-localizations of iron with amyloid beta were reported using an innovative method combining magnetic resonance imaging (MRI) and immunohistochemistry (Meadowcroft, Connor, Smith, & Yang, 2009). As discussed earlier, iron is recognized as a major cause of oxidative stress due to its potential in forming hydroxyl radicals from hydrogen peroxide, otherwise known as the Fenton Reaction (Honda, Casadesus, Petersen, Perry, & Smith, 2004). This hypothesis ties together the multifaceted nature of AD by connecting the development or presence of each of the typical pathologies of AD with iron dysregulation and increase in oxidative stress, which is why we have designated it the "Iron Hypothesis."

1.5 The Players

For over 100 years, the focus of AD research and treatment has been on two proteins, amyloid beta, and tau. Depositions of extracellular senile plaques, primarily mediated by Aβ, and development of intracellular neurofibrillary tangles have always been the hallmarks of this disease. This is rightly so, because these plaques and tangles in turn promote degeneration of neuronal synapses, which is progressive, and irreversible (Butterfield & Boyd-Kimball, 2004; Ittner et al.,

2010). These events are primarily seen in the neocortex, hippocampus and other subcortical regions involved in cognitive function (Moreira, Carvalho, Zhu, Smith, & Perry, 2010). The progression, however, is quite subtle as these pathological changes occur many years prior to manifestation of any clinical symptoms, making them ideal markers to predict AD, despite their elusive and intractable nature (Nordberg, 2008). Yet with the emergence of the iron hypothesis and related fields of study that connect the development of Aβ and tau to other metabolic systems, we may be able to better understand the pathogenesis, and breakthrough the barriers preventing effective diagnosis and treatment.

1.5.1 APP and Amyloid Beta

Aβ will invariably remain an integral part of AD research and therefore understanding it as completely as possible will always be paramount. The Aβ peptide is actually a normal physiological product from proteolytic processing of its progenitor protein APP; a transmembrane protein whose precise function is unknown, but its overexpression shows a positive effect on cell health and growth (O'Brien & Wong, 2011). Most APP proteins undergo normal proteolytic processing by α and γ-secretase respectively (non-amyloidogenic pathway), producing products promoting normal cellular functions. Those APPs that fail to be processed normally are cleaved by beta site APP cleaving enzyme (BACE) and γ-secretase (amyloidogenic pathway), generating Aβ (Greenfield et al., 1999; Younkin, 1998). Generally, such enzymatic actions produce Aβ fragments of variable length, with mostly 40 (Aβ40) and 42 (Aβ42) amino acid residues respectively. The most abundant residue is Aβ40, with only around 5-15% Aβ42 being produced (Durkin et al., 1999). Normally in young and healthy brains, these residues are tightly regulated (Shankar & Walsh, 2009), however, in old and pathological brains, the Aβ oligomers are shown to aggregate and initiate disease progression, promoting neuronal dysfunction (Bao et al., 2012;

Harkany et al., 2000; Qiu, Kivipelto, & von Strauss, 2009). PSEN1 and PSEN2 regulate enzymatic function of γ-secretase, and any defect or mutation in these genes leads to increased formation of Aβ as seen in early onset forms of AD (Ridge et al., 2013). Aβ has been connected to AD since its discovery, but other pathologies are equally important to understanding the disease.

1.5.2 Microtubule Associated Protein - Tau

One pathology that has been shown to be vital for Aβ induced neurotoxicity is tau protein (Roberson et al., 2007).Tau is a microtubule-associated protein important in the assembly and stabilization of microtubules (Weingarten, Lockwood, Hwo, & Kirschner, 1975). Tau also influences motor proteins like kinesin and dynein that regulate anterograde and retrograde axonal transport respectively (Stamer et al., 2002). Tau proteins are derived from a family of six isoforms, which range from 352 to 441 amino acids in length and differ only due to the presence of three or four repeat regions (3R,0;4R,0;3R,29;4R,29;3R,58;4R,58) found at the carboxyl terminus, and presence or absence of two peptide inserts at the amino terminus (Buee, Bussiere, Buee-Scherrer, Delacourte, & Hof, 2000). Three isoforms of tau containing exon 10 (4R) are more efficient at promoting microtubule assembly (Goedert & Jakes, 1990), while the isoforms that lack exon 10 (3R) are found to be associated with AD (Espinoza, de Silva, Dickson, & Davies, 2008). Unlike natively unfolded tau, aggregated tau proteins that form into paired helical filaments (PHFs) and into neurofibrillary tangles (NFTs) are associated with tauopathies which are characteristic of various neurodegenerative diseases, and especially AD (Lee, Goedert, & Trojanowski, 2001). Additionally, while tau is predominantly an intracellular protein, many recent experiments suggest physiological release of tau proteins into the extracellular spaces for potential seeding of pathogenic tau aggregates, allowing tau pathology to spread between neurons (Holmes & Diamond, 2014). Increased neuronal activity may be involved in regulating these processes and

stimulating this spread from neuron to neuron (Pooler, Phillips, Lau, Noble, & Hanger, 2013; Yamada et al., 2014).

1.5.2.1 Tau Hyper-phosphorylation

Another curious attribute of tau pathologies is the phosphorylation state of the proteins. Tau protein functions are regulated by phosphorylation state, and the tau proteins, found in the PHFs that form NFTs, are abnormally phosphorylated (Grundke-Iqbal, Iqbal, Quinlan, et al., 1986; Grundke-Iqbal, Iqbal, Tung, et al., 1986; Kosik, Joachim, & Selkoe, 1986). This hyper-phosphorylation disrupts normal function of tau, suggesting that its role in pathogenesis of tauopathies is to induce microtubule network breakdown leading to neuritic atrophy and neurodegeneration. Studies of tau in AD patients with tauopathies or in transgenic mice show hyper-phosphorylation of tau preceding aggregation (Braak, Braak, & Mandelkow, 1994). Additionally, iron in its oxidized state (Fe^{3+}) has been indicated to induce aggregation of hyper-phosphorylated tau pathologies, while the reduced ferrous iron (Fe^{2+}) reduces that same aggregation (Yamamoto et al., 2002). Following hyper-phosphorylation of tau, other notable losses in function include, but are not limited to: iron transport, neurogenesis, long term depression (LTD) and DNA protection (Chauhan & Chauhan, 2006; Lei et al., 2012; Sultan et al., 2011). Additionally, tau hyper phosphorylation has been linked with elevated levels of oxidative stress, neural connectivity problems, and elevated levels of plasma homocysteine, a key player in the iron hypothesis (Mondragon-Rodriguez et al., 2013; Vafai & Stock, 2002).

1.5.3 Homocysteine

Homocysteine (hcy) is known to be a neurotoxic non-protein amino acid (Kruman et al., 2000), and is also known to be elevated in AD (Miller, 1999). Its neurotoxicity is mediated via mechanisms such as stimulating NMDA (Lipton et al., 1997) and glutamate receptors (Kruman et

al., 2000), but is also known to mobilize iron from ferritin, preventing its storage and promoting iron-mediated oxidative damage (Dwyer, Raina, Perry, & Smith, 2004; Wagner et al., 2000). In addition to dysregulating iron, hcy has been implicated in contributing to tau protein hyper-phosphorylation through interaction with phosphatases, and Aβ production through a possible interaction with γ-secretase (Pacheco-Quinto et al., 2006; Vafai & Stock, 2002; Škovierová et al., 2016). Hcy is produced naturally in the body through metabolism of the essential amino acid methionine, but it is typically either converted back to methionine, or continues alteration into the amino acid cysteine and eventually the antioxidant glutathione (Brosnan & Brosnan, 2006; Stipanuk, 2004). In the presence of excess methionine, typically due to diet, or when the pathway is disrupted, however, systemic hcy levels will increase dramatically, raising the risk for a variety of diseases (Pettigrew et al., 2008). Dietary hormones and metabolites are therefore crucial in maintenance of normal physiological consequences for this pathway (Shea & Rogers, 2014). Enzymes in this pathway utilize several vitamin and mineral co-factors, including: vitamin B12, vitamin B6, folate, insulin and iron (Baggott & Tamura, 2007; Gursu, Baydas, Cikim, & Canatan, 2002; Shea & Rogers, 2014), indicating that diet and lifestyle could have a large impact on Alzheimer's disease.

1.5.3.1 Cystathionine β Synthase

One of the integral enzymes used in the metabolism of hcy is named Cystathionine β Synthase (CBS). In the transsulferation pathway, hcy is irreversibly converted into cystathionine through addition of a serine and catalyzed by CBS (Brosnan & Brosnan, 2006). The cystathionine product is then converted into the amino acid cysteine and then finally the antioxidant glutathione. The production of glutathione through the transsulferation pathway not only directly reduces hcy levels by hcy being its progenitor, but also directly through its antioxidant properties (Ovrebo &

Svardal, 2000). CBS is regulated via dietary hormones, which directly impacts the levels of hcy in the plasma and therefore has impacts on the oxidative stress levels in the cell. One such hormone is insulin, which tends to decrease CBS activity (Ratnam et al., 2002). In a study performed using rats, there was a discrepancy of insulin's impact on CBS activity based on the rat's diabetic development, i.e. if insulin-deficient diabetes was present, insulin decreased CBS activity while the inverse was true for diet-induced diabetic subjects (Tessari et al., 2005). The authors did however conclude that in general homocysteine disposal and clearance are impaired in type-2 DM patients, which generally presumes insulin resistance and elevated levels of insulin (Wilcox, 2005).Even more interesting than the connection between CBS and insulin is the apparent connection between CBS and iron. CBS possesses heme subunits, which will alter its activity state based on the redox potential of the heme group. Generally the activity of CBS doubles when the reduced Fe^{2+} heme group is oxidized to an Fe^{3+} group (Puranik et al., 2006). Zhou et. al. took the CBS/iron relationship a step further, stating that it was directly involved in systemic iron regulation (Zhou et al., 2018). Using a CBS double knockout, they saw massive iron dysregulation, anemia and elevated hepcidin levels. Under physiological conditions it is paramount that iron regulation be balanced, as both too much and two little can lead to increased oxidative stress and cellular catastrophe (Bresgen & Eckl, 2015).

1.6 The Proposal

As demonstrated by the above review there is an excess of pathways and contributing factors that confound and complicate the research and understanding of this terrible disease. We aim to better understand the role that iron plays in the development and progression of AD. Iron appears to be involved directly with both the diseases inception, as well as contributing to its continued advancement, but fortunately can act as a diagnostic marker as well point of attack in

treatments and prevention. The following aims outline how we intend on understanding each of these aspects of the disease.

1.6.1 Specific Aim 1: The Iron Hypothesis and its Effects on Aβ and Tau Aggregation as Seen in Murine Behavior, and Insulin Resistance

We will determine the mechanism by which the oxidative stress treatments of elevated methionine, and the western diet, affect the formation of tau tangles and amyloid plaques in WT, transgenic APP/PS1, and tau mice, and whether those formations are altered through antioxidant treatment with metformin. We will analyze memory performance (radial arm maze) at regular time intervals (2 [baseline], 3, 6, 9 Mo), and insulin resistance upon completion of the behavioral testing.

1.6.2 Specific Aim 2: The Iron Hypothesis and its Effects on Aβ and Tau Aggregation as Seen in T2-Star Turbo Spin Echo, 3D Gradient Echo and 3D UTE Cone sequences

We will determine the mechanism by which the oxidative stress treatments of elevated methionine, and the western diet, affect the formation of tau tangles and amyloid plaques in WT, transgenic APP/PS1, and tau mice, and whether those formations are altered through antioxidant treatment with metformin. We will analyze brain anatomy of our mouse cohorts using the following MRI scanning sequences: 2D T2* TSE, 3D GRE at 4.8 ms TE, and 3D UTE Cones at serial TE values of .3, .6, 1.2, and 2.4 ms.

CHAPTER 2: The Iron Hypothesis and its Effects on Aβ and Tau Aggregation as Seen in Murine Behavior, and Insulin Resistance

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2.1 Abstract

With the number of Alzheimer's disease (AD) patients increasing steadily every year, it is becoming more and more important that we determine the underpinnings of the disease so that we can diagnose and treat it more effectively. The Iron Hypothesis of AD pathogenesis suggests that the activity of certain iron response proteins could be potential biomarkers and/or targets for treatments. We tested this using a transgenic mouse model targeted specifically at APP, PSEN1 and tau genes. Using diet delivered stressing and rescue treatments we aimed to examine the changes that occurred in behavior using the radial arm maze test, and responses to an insulin injection during an insulin resistance test. We report that the transgenic mice performed significantly more poorly in the behavioral trials, despite rescue treatments. Western diet cohorts showed decrease cognitive ability in the radial arm maze task, while select cohorts with the rescue treatment showed significant attenuation of the cognitive decline. Female mice also performed more poorly in the maze tasks than their age matched male cohorts in the first behavioral trials. Additionally, metformin while a potential treatment should be better understood and diet monitored while on the drug. These results show that there is significant evidence backing the Iron Hypothesis, as a description to the progression of AD pathogenesis.

2.2 Introduction

Alzheimer's disease (AD) has been devastating families for well over a 100 years (Boller & Forbes, 1998). While much of the disease has been elucidated, we are still far from understanding how to predict and effectively treat those with AD (Casey, Antimisiaris, & O'Brien, 2010; Kong et al., 2015). The progression of the disease has been identified and categorized from several standpoints, but even the accuracy of those measures cannot be proven until after death (Beach, Monsell, Phillips, & Kukull, 2012; Braak & Braak, 1995). Even with the discovery of

several genes linked to AD, we still cannot explain the majority of cases (Bekris et al., 2010). The greatest correlating factor remains age, as 95% of AD patients are 65 or older. With the number of AD patients increasing steadily each year, it is becoming a larger financial burden on the world's population, as well as a psychological and emotional strain on those who live with and take care of patients stricken with the disease (Karlawish, Jack, Rocca, Snyder, & Carrillo, 2017).

2.2.1 A Change in Behavior

The first observation the family and friends of an Alzheimer's disease (AD) patient notice is the changed behavior of their loved one. While there are a wide variety of cognitive, behavior and psychological changes that occur within AD, one of the first, and definitely the most well-known, is altered memory (Li, Hu, Tan, Yu, & Tan, 2014; Neugroschl & Wang, 2011). This change in behavior is accounted for by the massive cell death, which occurs in the brain and leads to significant atrophy to the brain mass, which is visible through Magnetic Resonance Imaging (MRI) (de Leon, Bobinski, Convit, Wolf, & Insausti, 2001; de Leon et al., 1993; Jahn, 2013). The cause for this massive cell death is still under scrutiny, but involves some combination of tau and Aβ protein aggregation. Whether there is an underlying cause causing both the protein build up and cell death, or the cell death is a direct result of the buildup remains to be absolutely determined, but there is no doubt that when cell death occurs, whatever the cause, that a memory deficit follows (Gong & Iqbal, 2008; Gong et al., 2003; Malek-Ahmadi, Perez, Chen, & Mufson, 2016).

2.2.2 Animal Behavior Models

This same memory deficit has been recreated using a variety of assays in a number of different animal models, mostly using mice. With a number of maze options to choose from, including but not limited to, the Morris water maze, the Y-maze, the T-maze, the radial arm maze and even different combinations of each maze, we were forced to examine what constraints each

would put on our study (Chen et al., 2013; Byran, Lee, & Perry, 2009; Olton & Samuelson, 1976; Puzzo, Lee, Palmeri, Calabrese, & Arancio, 2014). Based on the number of subjects, the repetition of our trials each day and the length of the required testing periods we decided on the radial arm maze, which is well documented in animal behavior tests in general as well as specifically in AD models (Hodges, 1996; Wahl et al., 2017).

2.2.3 Aim of Experiments

In order to better understand the application of our iron hypothesis we proposed a handful of oxidative stress inducing diets and treatments aimed at elevating the reactive oxygen species (ROS) in our mouse cohorts, and ultimately dysregulating iron storage and metabolism within the cell. This dysregulation of iron and elevation of ROS would then, in theory, lead to an increase of Aβ plaque and tau tangle formation. We will be able to measure the formation of these plaques and tangles as they begin to effect the mouse subjects' cognition through differential behavior performance between treatment and genotype cohorts in the radial arm maze test. The results of these experiments will show the importance of iron regulation in AD development and progression and potential new treatment methods.

2.3 Materials and Methods

2.3.1 Animal Husbandry

To supply our behavior cohorts, we maintained and bred a mouse colony of several different mouse strains. Ordered from Jackson Laboratories and approved for experimental use by the BYU Provo Institutional Animal Care and Use Committee (IACUC), we maintained three strains in the BYU Life Science Building (LSB) Vivarium for cell culture, namely; C57BL/6J (WT), B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J (TAU), and B6;129-Psen1tm1Mpm-

Table 2.1: Genotype and Gender Cohorts.

Genotype and Gender By Experiment Table

Experiment	WT		WT Total	Tau		Tau Total	APP/PS1		APP/PS1 Total	PSEN1		PSEN1 Total
First RAM	4F	21M	25	10F	8M	18	16 F	11M	27	0 F	0 M	0
Second RAM	18F	6M	24	10F	16M	26	0F	0M	0	12F	12M	24
MRI	18F	6M	24	10F	16M	26	0F	0M	0	12F	12M	24

Tg(APPSwe,tauP301L)-1Lfa/Mmjax (PSEN1). During our first round of behavior collecting data, the PSEN1 mice were crossed with B6.Cg-Tg(APP695)3Dbo Tg(PSEN1dE9)S9Dbo/Mmjax (APP) giving us an APP/PSEN1 cross. Unfortunately, between the first and the following experiments the APP breeders had to be culled and despite ordering a new cohort of the APP mice we were not able to get the colony breeding in time to be included. As we still had PSEN1 mice, we used them without the cross. Each of the breeders was fed ad libitum both water and Teklad diets TD.8604 (Envigo, Madison, Wisconsin USA) supplied by the LSB vivarium. One male and one female were placed together in the male cage for 5 days and then separated back to their individual cages. Cohorts were sexed and separated at P21 and allowed to grow and mature. At 2 months of age they were started on the diet treatments, and began behavioral testing. All procedures involving animals were reviewed and approved by the BYU IACUC, ensuring that animals were treated humanely and in accordance to IACUC guidelines. The animals bred for the behavioral tests were also used for MRI scanning sequences upon completion of the following experiments.

2.3.2 Diet Treatments and Animal Cohorts

The cohorts were comprised of 144 subjects split between the different genotypes and treatment groups as well as the different time points involved. 70 mice were used for the first round of behavior and 74 mice were used for the second round of behavior. Additionally, there were several treatment groups within each cohort of the transgenic mice. Cohorts received either a stress

Table 2.2: Treatment Amounts and Groups. Each of the groups that received either a stress, a treatment or both are described above, along with the concentrations of each of the treatments. All groups except those denoted with a star were used in both sets of experiments.

*These treatments and groups were only used during the first RAM

**These treatments and groups were only used during the second RAM

Treatment Amounts and Groups Table

Treatments	Concentration (per g of chow)	Dosage
Methionine	10mg/g	1500mg/kg
Metformin	.66mg/g	100mg/kg
Clioquinol*	.20mg/g	30mg/kg
Zinc*	1.33mg/g	200mg/kg
Western Diet**	Fat - 21%, Sucrose - 32%	----------

Group #		Stress	Treatment
1	-	No Stress	No Treatment
2	-	No Stress	Metformin
3*	-	No Stress	Clioquinol
4*	-	No Stress	Zinc
5	-	Methionine	No Treatment
6	-	Methionine	Metformin
7*	-	Methionine	Clioquinol
8*	-	Methionine	Zinc
9**	-	Western Diet	No Treatment
10**	-	Western Diet	Metformin

treatment, a rescue treatment or both for the duration of the experiments, each with their own controls. The diets were custom ordered from Teklad (Envigo, Madison, Wisconsin USA) except for the western diet which is a premade diet (TD.88137). (see Table 2.2) As with the transgenic mice groups we had several differences between the first and second RAM experiments based on the results of the first and new directions. These differences in genotype and treatment can be seen on Table 2.1 and Table 2.2 respectively.

2.3.3 Behavioral Assays: Radial Arm Maze

Mouse behavior was tested using a custom made 8 arm Radial Arm Maze (RAM). Our protocol was designed similarly to Hodges radial arm maze protocol (Hodges, 1996). The radial arm maze, pictured in Figure 2.1, was constructed out of 7 mm thick opaque, white, plastic. The dimensions of the maze were a 27 inch (685.8 mm) diameter, 3.5 inch (88.9 mm) arm width, 8 inch (165.1 mm) arm height, 9 inch (228.6 mm) arm length, and 1 inch (25.4 mm) hole diameter at the end of each arm for food baiting (see Figure 2.1). The end of each arm had a unique symbol as a visual cue for the mouse. The maze was surrounded by a curtain to reduce spatial cues in the room and the varying undergraduate research associates positions and number. Mice were run in

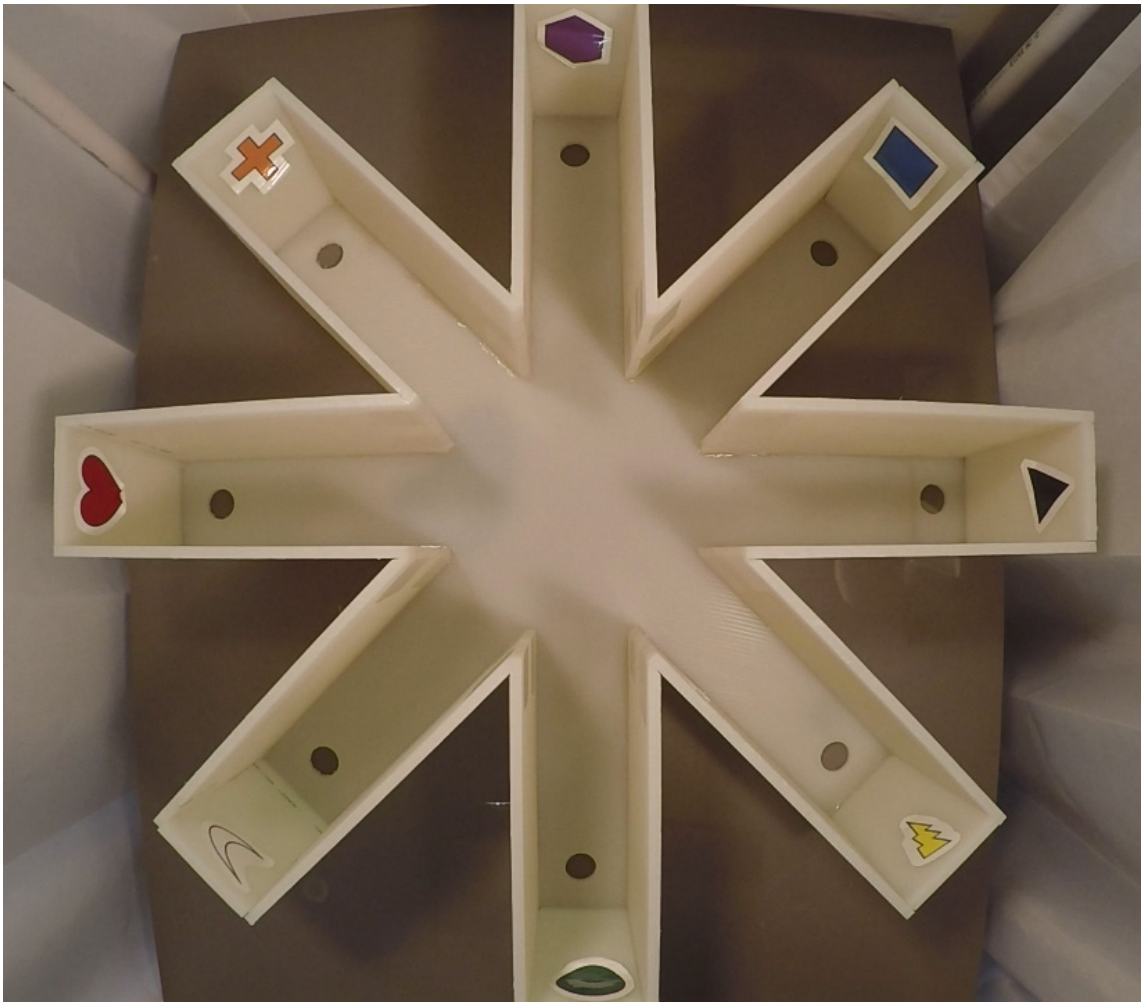

Figure 2.1: Radial Arm Maze Layout.

the maze at the testing periods of 1(baseline), 3, 6 and 9 months of age during the first tests and 2(baseline), 3, and 6 months during the following experiments. The change of baseline from 1 month of age to 2 months of age was due to lack of maturity, and negative effects on the mouse health due to fasting and stress of the maze at 1 month. The same negative effects were not observed when we transitioned to 2 months. Each testing period lasted for 2 weeks (10 weekdays total) and consisted of 10 trials a day. Each trial was run for 5 minutes (300 seconds) or until the mouse discovered the baited arm. During the initial RAM tests a specific arm was baited for each mouse until they reached completion criterion, which we determined to be entering the baited arm on the first try, 3 trials in succession. The arm was baited with roughly $1/8^{th}$ to $1/16^{th}$ of a cheerio during the first behavior experiments and an equal portion of the normal chow during the second experiments. After completion criterion, the mouse's baited arm would switch to a reversal arm, and testing continued until reaching completion criterion again. This was repeated for the duration of the testing periods. During the second round of behavioral tests however, baited arms were only changed after a testing period had concluded to help with analysis and comparison of the videos. This ensured that all the mice had the same number of trials in the same number of arms, which allowed us to compare across and within cohorts, but unfortunately prevented us from comparing across the two different sets of behavior experiments. The setup of our maze emphasized the visual cues rather than spatial cues, both of which seem to have overlapping effects on memory processing and formation (Vorhees & Williams, 2014). Olton et. al. showed that ablation of fimbria-fornix, decreased performance to the working memory of a task where no cues were present, and Ward et. al. found similar deficiencies in mice with ablated hippocampus in both spatial and visual cue tasks (Olton & Paras, 1979; Ward, Stoelzel, & Markus, 1999).

2.3.4 Behavioral Analysis

The videos were analyzed using ANY-maze Behavioral Tracking Software (Stoelting Co.; Wood Dale, IL; version 4.99m). We recorded time spent in the maze, distance run during a trial, and number of errors made, based on the number of times the mouse entered an incorrect (not baited) arm, and during the second set of behavioral experiments recorded the number of failed trials. Statistics on each of those values were calculated using SPSS by performing a multivariate ANOVA and correcting for multiple comparisons using a Tukey HSD correction.

2.3.5 Insulin Resistance Test

After each mouse completed its behavioral testing it was subjected to an insulin resistance test, which examined the animal's physiological response to an IP injection of insulin. The mice were fasted for 6 hours before performing the test. Each mouse was weighted and based on their weight were given 0.75 IU/kg of Novolin R Insulin (Novo Nordisk, Bagsværd, Denmark), and glucose levels were measured at -15, 0, 15, 30, 60, 90 and 120 minutes from time of injection. Glucose levels were measured using the Accu-Chek Guide meter and accompanying Accu-Chek Guide test strips (Roche Diabetes Care Inc., Indianapolis, Indiana, USA), and acquiring blood from a very small cut on the tip of the tail.

2.4 Results

2.4.1 First Behavior Experiments

The behavior results will be split into two different sections because, despite the fact that we used the same mice, maze, and general protocol, we altered the completion criteria and therefore how the data was analyzed, making them essentially two separate experiments. A caveat to the first results is that there were reduced numbers of mice in each of the cohorts which reduces the statistical power of the experiment. With 2 oxidative treatments, 3 rescue treatments and 3 time

points, our cohorts were left with only 1 or 2 mice each. In the future we hope to repeat the experiment and focus in on select cohorts thereby increasing the number of mice per group and hopefully the power as well. We also plan to continue the study further by examining the deposition of the Aβ proteins and plaques and tau tangles, among other proteins, through histology, western blot and NMR.

2.4.1.1 Time in RAM

Each mouse was placed in the RAM and given a total of 5 minutes (300 seconds) to explore the different arms and discover the hidden treat in the baited arm. If the mouse found the baited arm the trial was ended immediately. We saw a statistically significant main effect of genotype [F(2,25)=13.505, P=0.000] and a trend towards significance for genotype and treatment interaction

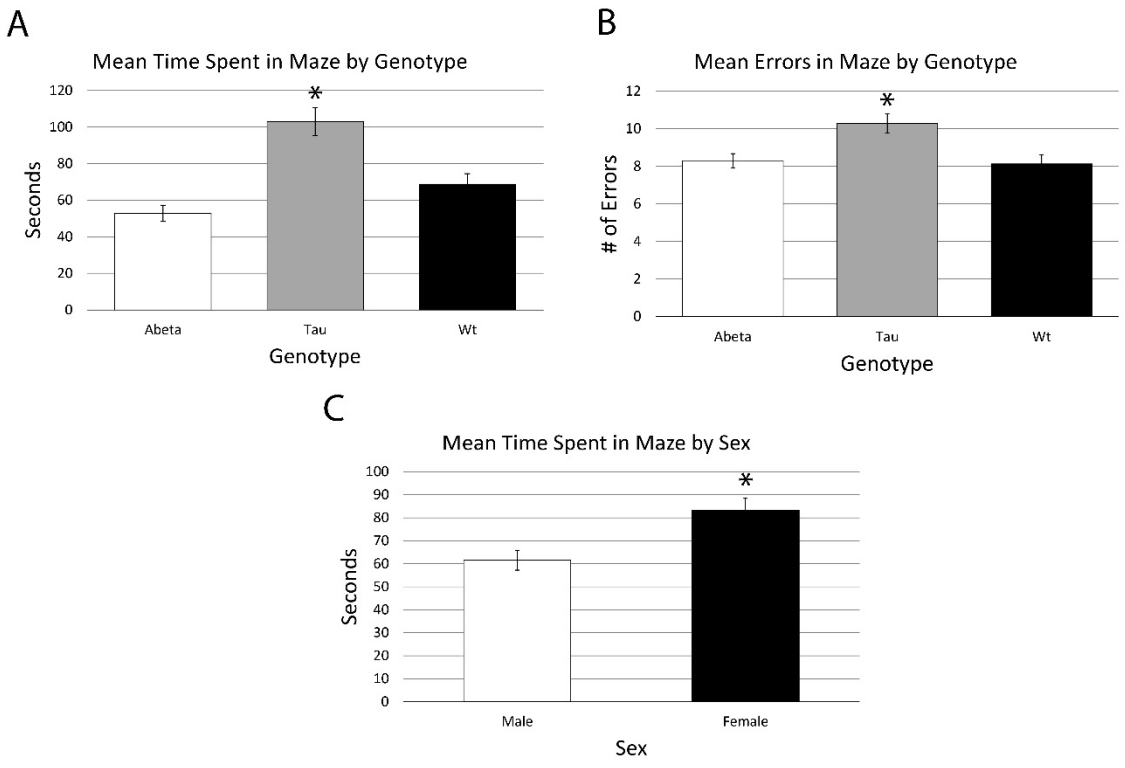

Figure 2.2: First RAM Behavior Results. (A) Tau mice spent more time in the maze than WT cohorts as well as APP/PS1 cohorts. (B) Tau mice made more errors than the other cohorts of mice. (C) Female mice spent more time on average than the male mice.

[F(11,23)=1.900, P=0.094]. Post hoc analysis of the genotypes showed that WT and tau had a mean difference of the time spent in the maze of 34.225 [+/- 9.607: p = 0.003] and surprisingly APP and tau had a mean time difference of 50.167 [+/- 8.913; p = 0.000]. (Figure 2.2-A) Female mice spent a mean time of 21.745 seconds [+/-6.726: p = 0.002] longer in the maze then the males in the study. (Figure 2.2-C)

2.4.1.2 Distance in RAM

The entire distance that a mouse ran while being tested in the RAM was measured and compared across and between the cohorts. Fixed effects of genotype showed no significant difference (p = 0.418), but pairwise comparisons of genotype revealed a trend towards significance between the WT and Tau cohorts with a mean difference of .662 (+/-.273: p = 0.062). Pairwise comparisons between male and female subjects showed not significant difference (p = 0.141), and comparisons between treatment reveals no trends or significance as well (p > 0.571).

2.4.1.3 Errors in RAM

An error was calculated each time that a mouse entered an incorrect (unbaited) arm during the duration of each of a mouse's trials. There was a trend towards significance for genotype in number of errors made [F(2,10)=3.377, P=0.074]. Post hoc analysis of the genotypes showed that WT and tau had a mean difference of the number of errors of 2.144 (+/- 6.95: p = 0.015) and again surprisingly APP and tau had a mean error difference of 1.973 (+/- .635: p = 0.024). (Figure 2.2-B) As with what was seen measuring subject mean time spent in the maze, female mice had a trend toward significance in mean errors with a mean difference of 1.012 (+/-.512: p = 0.064).

2.4.2 Second Behavior Experiments

2.4.2.1 Time in RAM

The total time for each mouse was recorded from the time released to either the moment the subject entered the baited arm or five minutes elapsed. There was a statistically significant difference in time spent in the maze based on the treatment administered, $F(15, 8.683) = 15.183$, $p < 0.0005$; Wilk's $\Lambda = 0.000$; based on the genotype of the mouse, $F(6,6) = 32.255$, $p < 0.0005$; Wilk's $\Lambda = 0.001$; based on the sex of the mouse, $F(3,3) = 89.156$, $p = 0.002$; Wilk's $\Lambda = 0.011$; as well as a statistically significant interaction effect between treatment administered and genotype, $F(24,9.302) = 6.126$, $p = 0.003$; Wilk's $\Lambda = 0.000$; as well as between treatment administered and the sex of the mouse, $F(15,8.683) = 8.834$, $p = 0.001$; Wilk's $\Lambda = 0.000$. Significant differences with $p = 0.012$ and less, for the above mentioned conditions (treatment, genotype and sex) were only seen in the 3 month and 6 month testing stages, and not in the baseline behavioral tests. A Tukey HSD post hoc test revealed that during the 3 month testing period the group 1 control diet

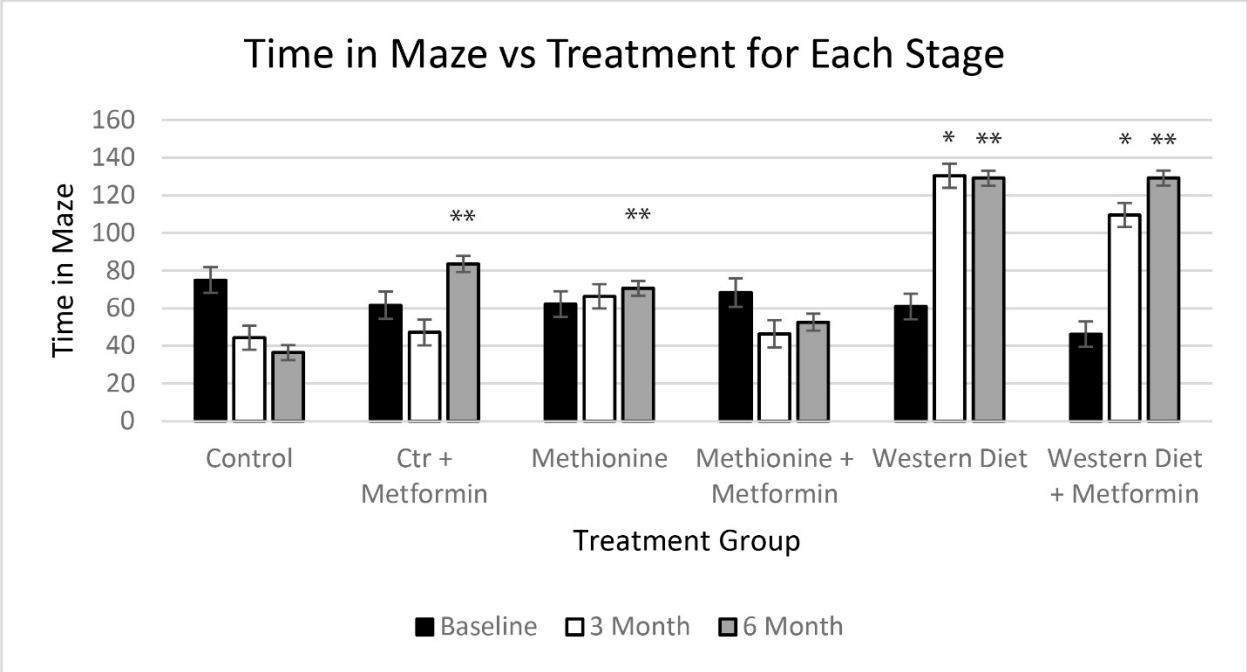

Figure 2.3: Mean Time in Maze vs Treatment for Each Stage.

mean time was significantly different from both the group 9 [western diet] (-85.274 +/-8.687 sec, $p = 0.001$) and group 10 [western diet + metformin] (-62.881 +/- 8.687 sec, $p = 0.005$). The other 3 month treatment groups showed no significant difference from the control diets. The trend continued to become more pronounced in the 6 month testing period. Mean time spend in the maze became significantly different between group 1 and groups 2 [control + metformin] (-48.659 +/- 5.692 sec, $p = 0.003$), 5 [increased methionine] (-33.635 +/- 5.367 sec, $p = 0.01$), and again 9 (-84.798 +/-5.367 sec, $p < 0.0005$) and 10(-84.743 +/-5.367 sec, $p < 0.0005$). There was no statistically significant difference between the control diet and group 6 [increased methionine + metformin] ($p = 0.311$). Post hoc tests also revealed a significant difference between WT mice and PSEN1 cohorts at all three time points whereas there was no difference present between WT and tau cohorts, counter to what was seen previously in the first round of RAM trials.

2.4.2.2 Distance Run in RAM

The path that the mouse took through the maze was traced and recorded in order to give us a measure of the total distance that the mouse ran while being tested. There was a statistically significant difference in distance run while in the maze based on the treatment administered, $F(15, 8.683) = 7.309$, $p = 0.0003$; Wilk's $\Lambda = 0.001$; based on the genotype of the mouse, $F(6,6) = 7.504$, $p = 0.014$; Wilk's $\Lambda = 0.014$; based on the sex of the mouse, $F(3,3) = 22.901$, $p = 0.014$; Wilk's $\Lambda = 0.042$; as well as a statistically significant interaction effect between treatment administered and genotype, $F(24,9.302) = 3.125$, $p = 0.037$; Wilk's $\Lambda = 0.002$; but no significance between treatment administered and the sex of the mouse, ($p = 0.558$). Significant differences with $p < 0.005$, for the above mentioned conditions (treatment, genotype, sex and interactions) were seen in the 6 month testing stages. A Tukey HSD post hoc test revealed that during the 6 month testing period the group 1 control diet mean distance was significantly different from both the group 9 [western diet]

(-1.355 +/-.091 meters, $p < 0.0005$) and group 10 [western diet + metformin] (-.817 +/- .091 meters, $p = 0.002$). The other treatment groups showed no significant difference from the control diet. When comparing diet group 10 [western diet + metformin] to group 9 [western diet] there was a significant decrease in distance run in the maze (-.538 +/- .091 meters, $p = 0.013$). Post hoc tests also revealed a significant difference in mean distance between WT mice and PSEN1 cohorts at the 6 month time point whereas there was no difference present between WT and tau cohorts, again, counter to what was seen previously in the first round of RAM trials.

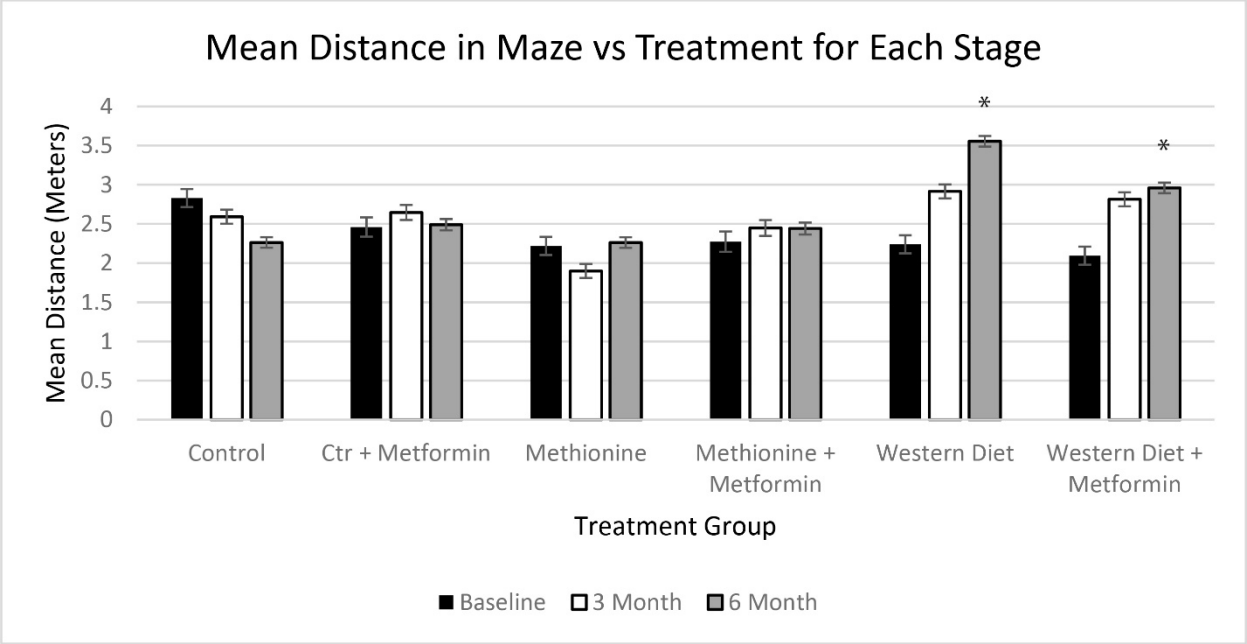

Figure 2.4: Mean Distance in Maze vs Treatment for Each Stage.

2.4.2.3 Errors in RAM

With the arms of the maze designated, the ANY-maze™ software calculated the number of errors that were made by each of the subjects during their trials. We found that there was a statistically significant difference in number of errors made while in the maze based on the treatment administered, $F(15, 8.683) = 6.613$, $p = 0.0004$; Wilk's $\Lambda = 0.001$; based on the genotype of the mouse, $F(6,6) = 13.233$, $p = 0.003$; Wilk's $\Lambda = 0.005$; based on the sex of the mouse, $F(3,3)$

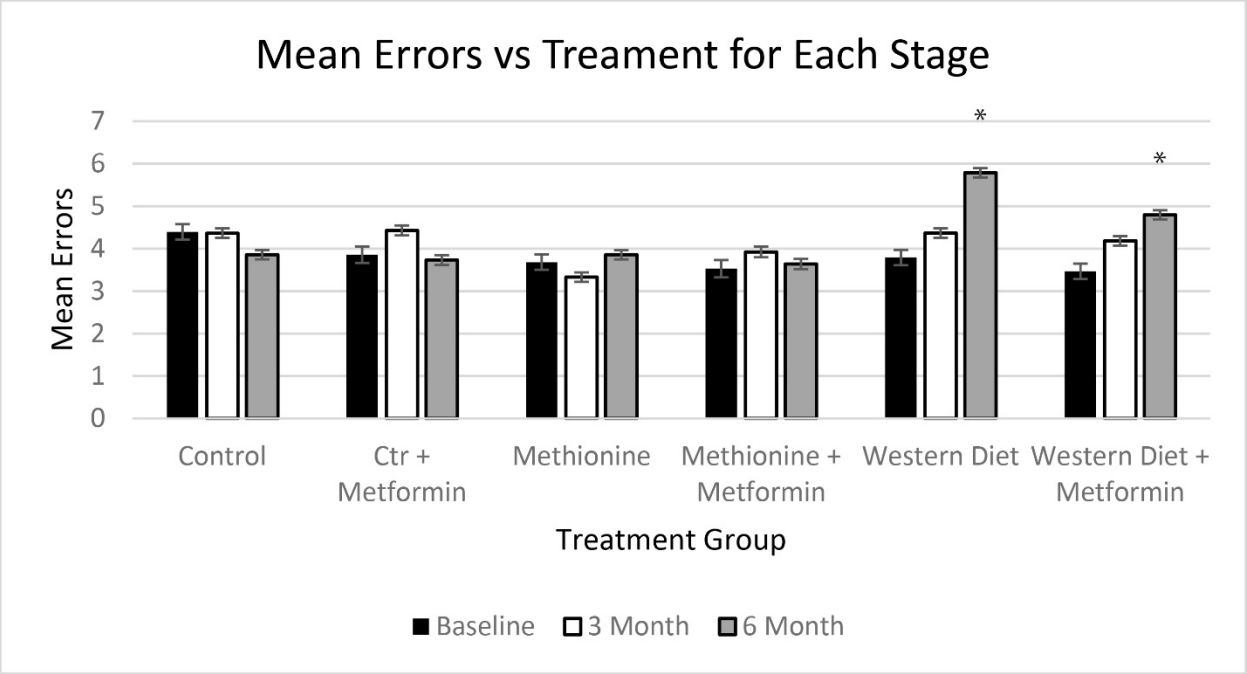

Figure 2.5: Mean Errors vs Treatment for Each Stage.

= 12.126, p = 0.035; Wilk's Λ = 0.076; as well as a statistically significant interaction effect between treatment administered and genotype, F(24,9.302) = 3.256, p = 0.033; Wilk's Λ = 0.002; but only a trend towards significance between treatment administered and the sex of the mouse, (p =0.076). Significant differences with p = 0.015 and less, for the above mentioned conditions (treatment, genotype, sex and interactions) were seen in the 6 month testing stages. A Tukey HSD post hoc test revealed that during the 6 month testing period, the group 1 control diet mean errors was again significantly different from both the group 9 [western diet] (-1.976 +/-.146 errors, p < 0.0005) and group 10 [western diet + metformin] (-1.178 +/- .146 errors, p = 0.003). The other treatment groups showed no significant difference from the control diet. When comparing diet group 10 [western diet + metformin] to group 9 [western diet] there was a significant decrease in mean errors in the maze (-.798 +/- .146 errors, p = 0.013). Post hoc tests also revealed a significant difference in mean distance between WT mice and PSEN1 (.782 +/- .096 errors, p = 0.001) as well as Tau (-444 +/-.124 errors, p = 0.036) cohorts at the 6 month time, although the PSEN1 mice

made significantly fewer errors than the WT and Tau mice, counter to the trends in the time and distance means.

2.4.2.4 Failed Trials in RAM

Since we were no longer utilizing the completion criterion, the opposite measure, or failure to find the baited arm i.e. timing out at 5 minutes, was recorded and analyzed. There was a statistically significant difference in the mean failed trials during a testing period based on the treatment administered, $F(15, 8.683) = 13.333$, $p < 0.0005$; Wilk's $\Lambda = 0.000$; based on the genotype of the mouse, $F(6,6) = 50.543$, $p < 0.0005$; Wilk's $\Lambda = 0.000$; based on the sex of the mouse, $F(3,3) = 107.056$, $p = 0.002$; Wilk's $\Lambda = 0.009$; as well as a statistically significant interaction effect between treatment administered and genotype, $F(24,9.302) = 10.205$, $p < 0.0005$; Wilk's $\Lambda = 0.000$; as well as between treatment administered and the sex of the mouse, $F(15,8.683) = 11.370$, $p = 0.001$; Wilk's $\Lambda = 0.000$. Significant differences with $p < 0.005$, for the above

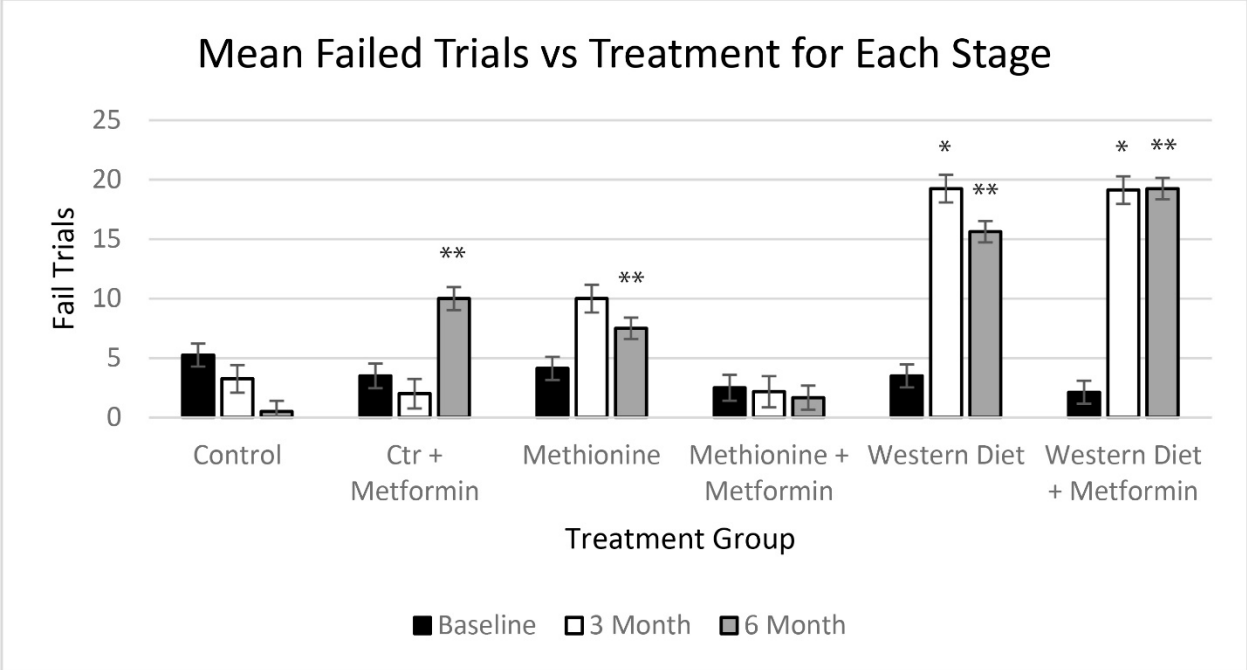

Figure 2.6: Mean Failed Trials vs Treatment for Each Stage.

mentioned conditions (treatment, genotype and sex) were seen in the 3 month and 6 month testing stages, and not in the baseline behavioral tests. A Tukey HSD post hoc test revealed that during the 3 month testing period the group 1 control diet mean time was significantly different from both the group 9 [western diet] (-14.60 +/-1.575 failed trials, $p = 0.002$) and group 10 [western diet + metformin] (-13.00 +/- 1.575 failed trials, $p = 0.003$). The other 3 month treatment groups showed no significant difference from the control diet. As was seen in the mean time the trend continued to become more pronounced in the 6 month testing period. Total failed trials became significantly different between group 1 and groups 2 [control + metformin] (-9.60 +/-1.290 failed trials, $p = 0.005$), 5 [increased methionine] (-5.80 +/- 1.217 failed trials, $p = 0.032$), and again 9 (-12.60 +/- 1.217 failed trials, $p = 0.001$) and 10 (-15.20 +/-1.217 failed trials, $p < 0.0005$).There was no statistically significant difference between the control diet and group 6 [increased methionine + metformin] ($p = 0.943$), but was a trend towards significance between group 6 [increased methionine + metformin] and group 5 [increased methionine] (-4.70 +/-1.290, $p = 0.089$). The same trend as other measures was present with fail trials in the 6 month stage between WT mice and PSEN1 cohorts (-14.89 +/- .803, $p < 0.0005$), but once again not between WT and Tau cohorts.

2.4.3 Insulin Resistance Test

After each cohort of mice finished their behavioral testing, but before they were scanned in the MRI, they were tested for insulin resistance. We found a statistically significant difference in the levels of glucose in the blood of the mice based on genotype $F(14, 38) = 4.793$, $p < 0.0005$; Wilk's $\Lambda = 0.131$; based on sex, $F(7, 19) = 3.332$, $p = 0.017$; Wilk's $\Lambda = 0.449$; based on treatment group, $F(35, 82.356) = 2.008$, $p = 0.005$; Wilk's $\Lambda = 0.075$; based on age of performing the procedure, $F(7, 19) = 3.725$, $p = 0.011$; Wilk's $\Lambda = 0.422$; as well as a statistically significant interaction effect between sex and diet treatment group, $F(35, 82.356) = 1.627$, $p = 0.037$; Wilk's

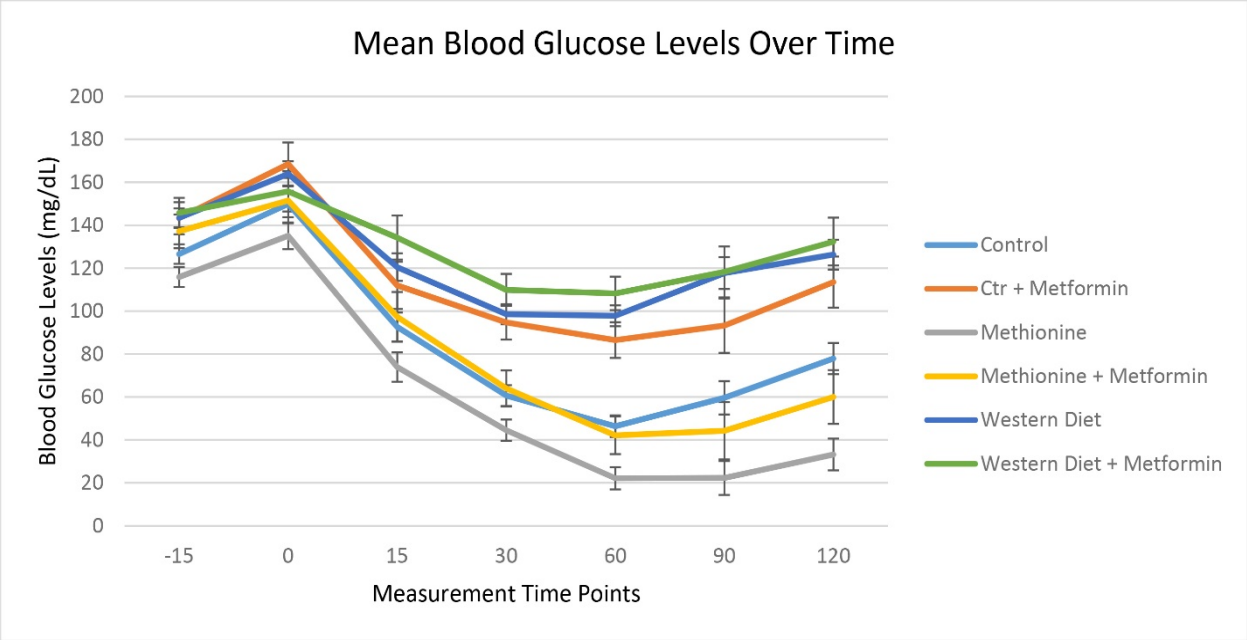

Figure 2.7: Mean Blood Glucose Levels Over Time.

$\Lambda = 0.110$. While there were significant contributions scattered across all the glucose measurement time points for each of the above mentioned variables, the most interesting post hoc Tukey HSD test results are with genotype at 30 minutes after injection and beyond ($p < 0.006$), and across every measurement time point for diet treatment group ($p < 0.006$). At 15 minutes until insulin injection there is almost no significant difference between the mean glucose levels for each of the treatments. This holds for time point 0, when insulin was injected. At 30 minutes after injection however, separation between group 1 [control] and groups 2 [control + metformin] (-28.96 +/- 9.020, $p = 0.038$), 9 [western diet] (-24.38 +/- 5.669, $p = 0.003$), and 10 [western diet + metformin] (-45.01 +/- 8.289 become readily apparent. This continues at the 60 minute measurement, but surprisingly at that point the treatment group 5 [increased methionine] is significantly lower than the control group (23.08, +/- 6.40, $p = 0.015$). The inverse relationship of the western diet groups and the methionine groups maintains through the final measurement at 120 minutes with a mean difference between group 1 and groups 5 (42.07 +/- 9.163, $p = 0.001$) , 9 (-30.37 +/- 8.478, p =

0.016) and 10 (-56.93 +/- 12.395, $p = 0.001$). Performing a univariate analysis of variance we find that there is a statistical significance for the amount of insulin injected at time point 0 for genotype ($p < 0.0005$), gender ($p < 0.0005$), diet ($p < 0.0005$), and age at time point 0 ($p < 0.0005$).

2.5 Discussions

2.5.1 First Round of Behavior

One of the first evidences of neuropathological changes that patients and the family members of those afflicted with AD notice is altered behavior; for this reason behavior and memory have become a widely tested measure in the many models of AD (Puzzo, Lee, Palmeri, Calabrese, & Arancio, 2014). We observed this same behavioral degeneration in our tau cohorts regardless of whether they were receiving the rescue treatment or not, but surprisingly the APP mice performed just as well as the WT mice. Aβ plaques have long been the culprit for the loss of memory and decrease in cognition, but recently amyloid has been shown to have varying effects on the memory and cognition in both humans and animal models (Van Bergen et al., 2016). The variation may come from differing plaque types, solubility of the Aβ peptides, and location of the deposition in the brain (Malek-Ahmadi et al., 2016; Van Helmond, Miners, Kehoe, & Love, 2010). Rogalski et. al. even examined 10 "SuperAgers", who they defined as people over the age of 90 who test at a cognitive level of an average person age 50-60. Upon autopsy, up to half of these "SuperAgers" had moderate to high densities of neuritic Aβ plaques, yet had above average cognitive performance (Rogalski et al., 2018). Another study using APP/PS1 transgenic mice found that treatment with estrogen improved spatial learning tasks in both the T-maze and the RAM, while not affecting the Aβ accumulation and plaque formation (Heikkinen et al., 2004). Our lack of difference in memory tasks might be attributed to these same variations in the Aβ peptides and plaques.

Variation of AD between sex has also become a heavily debated subject between dementia researchers. While most sources would state that there is roughly a 3:2 disparity between women and men with AD, some sources say that the difference only appears in the higher age groups of AD patients ("2016 Alzheimer's disease facts and figures," 2016; Mazure & Swendsen, 2016; Ruitenberg, Ott, van Swieten, Hofman, & Breteler, 2001). When looking specifically at visuospatial memory tasks, men routinely perform better than women with or without AD (Millet et al., 2009). Examining mouse studies there is hardly a consensus about the performance of transgenic and non-transgenic mice, with evidence of either gender outperforming the other, or having equal results (Clinton et al., 2007; Gimenez-Llort et al., 2010; Stevens & Brown, 2015). We add our results to the handful of mouse models and the human studies that show decreased female performance compared to males. The reason for this difference is less clear as we showed no significant difference of T2 signal decrease between the mice based on sex, despite the overall levels for both sexes of transgenic mice being decreased due to increased plaque deposition.

2.5.2 Second Round of Behavior

The main difference between our first set of behavioral trials and the second set was when the baited arm was changed. Initially we waited for completion criterion to be met, but that never happened for some mice, and thus made comparing across and within cohorts very difficult. To aid in our comparison we decided to change the arm at each testing period, regardless of whether completion criterion was completed or not. The decision to make the alteration was not arrived upon lightly as this meant that few, if any, comparisons could be made between the two experiments. Ultimately, the decision was made because whatever way we performed the trials time in the maze, total number of errors and distance were going to be our best measures. Additionally, we added a new diet with the western diet, which proved to have a massive impact

on the behavior in the RAM. At almost every single measure the western diet was impairing the ability of the subject to perform. Time spent in the maze increased significantly, distance run increased significantly, the number of errors increased significantly and the number of failed trials increased significantly as well. This is confirmed in multiple studies as well, with mice exhibiting decreased performance in the radial arm maze task when fed a high-fat or "western diet" (Cordner & Tamashiro, 2015). Alzoubi et. al. have even, on multiple occasions, demonstrated an ability to improve cognitive performance in the RAM using the diet interventions vitamin E and caffeine (Alzoubi, Abdul-Razzak, et al., 2013; Alzoubi, Khabour, Salah, & Hasan, 2013). Our own rescue diet of metformin showed promise in improving at least some of the measures when combined with a stress treatment, as was the case when combined with methionine and western diets during the 6 month trial periods, evidenced by decreased distance run, errors made and trials failed. There did seem to be an adverse effect on the treatment group 2, which receive metformin supplemented control diet. That cohort had increased times in the maze as well as increased failed trials during the 6 month testing period even after having performed commensurately to the control group during the baseline and 3 month stages. While metformin has been shown to attenuate Alzheimer's disease-like neuropathology in multiple publications, typically at twice the dosage of our experiments, its benefit for cognition is less established, although it has shown improvement in the Morris water maze (Li, Deng, Sheng, & Zuo, 2012; Ou et al., 2018). In 2017, Thangthaeng et al, published a paper citing the deleterious effects of a 200-300 mg/kg metformin treatment on their aged C57BL/6 mice (Thangthaeng et al., 2017). While this matches somewhat what was seen in our behavioral data, it also aligns with the discrepancies seen with the insulin resistance test.

2.5.3 Insulin Test

The results of the insulin resistance test were quite obvious when looking at the difference between the control and western diet cohorts. The western diet induced insulin resistance, and even our treatment with metformin did little to nothing to prevent the lack of response to the shot of insulin. Interestingly Figure 2.7 shows diet treatment group 2 following a similar trend as the western diet treatments, which is counter intuitive, as it was a control for the metformin treatments, and was not expected to deviate significantly. While there is extensive research showing the "miraculous" ability of metformin to attenuate insulin resistance in diabetics and PCOS patients, metformin's mechanism of action and interactions may shed light on the limits of its ability (Diamanti-Kandarakis & Dunaif, 2012). Metformin works by reducing the release of glucose from the liver, and potentially increase peripheral glucose utilization (Todd & Florez, 2014). Metformin has also be linked to vitamin B12 and folate deficiency with several independent studies, which intern dysregulates the methionine-homocysteine metabolic pathway, which has been connected to increased oxidative stress, inflammation, and ultimately diabetes and AD (Ahmed, Muntingh, & Rheeder, 2017; de Jager et al., 2010; Kibirige & Mwebaze, 2013). Thus in certain situations where diet is not monitored, and metformin is not kept in check, it has the potential to cause exactly what it has been show so effective at treating, especially in the diabetic and obese population. Weight also happened to be a large contributing factor in the correlations of the insulin resistance test. Our univariate analysis showed significance with treatment, genotype, sex and age of the mice, all of which influence the weight of the mouse. The diets impacted the cohorts the most significantly, but sex and age also contribute to the size and weight of the mouse.

2.6 Conclusions

The transgenic model presented shows the impact that diet and genotype can have on the development of Alzheimer's like pathology. The western diet has a direct effect on the decline of cognitive performance of this transgenic murine model as evidenced by increased RAM times, distances, errors and failed trials. PSEN1 transgenic mice exhibit Alzheimer's like pathology in decreased cognitive ability. Metformin as a treatment has significant potential if it is accompanied with diet monitoring, and a pre-existing diseased state. Further investigation is warranted to understand the drugs impact on the nervous system and varying conditions that accompany AD. The weakness of this study is numbers in each cohort. Narrowing the focus even further would allow for more subjects to be studied in the same amount of time and stronger correlations to be made. We have significant amounts of tissue for future as we collected brain tissue, ocular tissue and visceral tissue from each of the mice upon being euthanized. The brain was dissected and the left hemisphere was frozen for homogenization and western blot analysis. The right hemisphere, right eye and viscera were also saved and fixed for future analysis in histology as well as many other potential experiments.

2.7 Acknowledgements

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CHAPTER 3: The Iron Hypothesis and its Effects on Aβ and Tau Aggregation as Seen in T2-Star Turbo Spin Echo, 3D Gradient Echo and 3D UTE Cone sequences

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3.1 Abstract

Alzheimer's disease (AD) causes tremendous damage to the brain over the course of the life of those affected by the disease. A major focus of AD research pertains to the development of diagnostic tools and methods to examine the minute changes that occur early on within the brain in attempts to initiate earlier prevention and treatment for the disease. While there are many methods currently under investigation one of the most powerful currently is MRI. We aimed to extend the use of our transgenic mouse model and investigation of the Iron Hypothesis using oxidative stressors and antioxidant treatments to novel MRI scanning sequences in order to develop a sensitive diagnostic tool for AD development and progression. Using a combination of traditional T2 scan sequences (T2* TSE and 3D GRE) and specially developed 3D UTE Cone sequences, we scanned the same mice from our behavioral assays upon completion of their radial arm maze trials at 3 and 6 months. Experimental UTE MRI scanning sequences revealed variable signal intensities in the hippocampi of the transgenic mice based on significant signal differences in the 1.2 ms UTE scans for the western diet treatment cohorts and western diet with Metformin rescue cohorts. The scans require further post processing, and the techniques may require further adjustments, specifically for fat suppression

3.2 Introduction

One of the most frightening aspects of Alzheimer's disease is the uncertainty it creates for everyone, regardless of age, or familial history. Nobody know who may or may not develop the disease and there are little to know symptoms until the disease has caused extensive and irreversible damage to the brain. It is apparent, however, that the progression of the disease is occurring slowly and steadily for up to decades (Braak, Thal, Ghebremedhin, & Del Tredici, 2011).

Unfortunately, the amount of change that takes place is so slow and minute in most cases that it is near impossible to tell the difference between a diseased brain and a health brain.

3.2.1 MRI: The Diagnostic Tool

The uncontrollable nature of the disease abounds because medical professionals and researchers can only detect its presence in an individual after the progression has reached severely damaging stages of AD. One of the very few methods that exists for diagnostic and research purposes is MRI. Currently, however, MRI is not a perfect tool for diagnosing AD, but it does allow for visualization of neuroatrophy and other artifacts not normally observable until after autopsy (Frings et al., 2014; Johnson, Fox, Sperling, & Klunk, 2012; Vemuri & Jack, 2010). Unfortunately, many of those artifacts are still not visible using MRI until the advanced stages of AD, and therefore too late for effective preventative care. At advanced stages of AD, increased MR signal dropout is seen localized in areas of Aβ plaque and tau tangle deposition, linking those pathologies to clusters of iron deposits (Meadowcroft, Peters, Dewal, Connor, & Yang, 2015). Both of these proteins are found to have a strong affinity to iron, (Bouras et al., 1997; LeVine, 1997; Meadowcroft et al., 2009; Wan et al., 2011; Zheng et al., 2009) and since these iron deposits can be visualized using MRI we might be able to detect AD before it noticeably affects cognition. Low concentrations of early forming iron deposits are still not visualized in traditionally used T2 scans due to the very rapid relaxation time of the iron associated molecules, however, using ultra short echo times (UTE) of less than 1ms, the signal from the iron deposits can be captured (Robson, Gatehouse, Bydder, & Bydder, 2003; Sheth et al., 2017; Tyler, Robson, Henkelman, Young, & Bydder, 2007).

3.2.2 Aim of Experiments

The UTE sequence is becoming a popular method for imaging structures normally drowned out by the "louder," longer signals of hydrogen rich tissues. We aim to apply this technique to our transgenic mouse cohorts after they have been exposed to the oxidative stress inducing treatments, and completed measures in the other experiments for behavior and insulin resistance. We will use serial timing of the TE for each scan to test for an optimum range for scanning the brains and detecting the changed levels of iron with in the hippocampus. The results of these experiments will validate UTE as a diagnostic method for detecting minute changes in levels of iron within the brain, and contribute to understanding the application of this tool in human subjects.

3.3 Materials and Methods

3.3.1 Animal Husbandry

We maintained and bred a mouse colony of several different mouse strains. Ordered from Jackson Laboratories and approved for experimental use by the BYU Provo Institutional Animal Care and Use Committee (IACUC), we maintained three strains in the BYU Life Science Building (LSB) Vivarium for cell culture, namely; C57BL/6J (WT), B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J (TAU), and B6;129-Psen1tm1Mpm-Tg(APPSwe,tauP301L)-1Lfa/Mmjax (PSEN1). Each of the breeders was fed ad libitum both water and Teklad diets TD.8604 (Envigo, Madison, Wisconsin USA) supplied by the LSB vivarium. One male and one female were placed together in the male cage for 5 days and then separated back to their individual cages. Cohorts were sexed and separated at P21 and allowed to grow and mature. At 2 months of age they were started on the diet treatments, and began behavioral testing. All procedures involving animals were reviewed and approved by the BYU IACUC, ensuring that animals were treated humanely and in

accordance to IACUC guidelines. The mice used in the MRI experiments were tested behaviorally before being scanned and sacrificed.

3.3.2 Diet Treatments and Animal Cohorts

The cohorts were comprised of 74 subjects split between the different genotypes and treatment groups as well as the different time points involved. Additionally, there were several treatment groups within each cohort of the transgenic mice. Cohorts received either a stress treatment, a rescue treatment or both for the duration of the experiments, each with their own controls. The diets were custom ordered from Teklad (Envigo, Madison, Wisconsin USA) except for the western diet which is a premade diet (TD.88137). (see Table 2.2) As with the transgenic mice groups we had several differences between the first and second RAM experiments based on the results of the first and new directions. These differences in genotype and treatment can be seen on Table 2.1 and Table 2.2 respectively.

3.3.3 MRI Scanning Sequences

The sequences were developed for a 3T Siemens Trio TIM whole-body scanner (Siemens Healthineers, Erlangen, Germany) using a 4-turn Transmit/Receive (TR) solenoid radio frequency mouse coil manufactured by DOTY Scientific (see Figure 3.1). The mouse coil required manual shimming, and after the results were calculated the receiver gain was set to low and the FFT scale factor was set at 0.3. We acquired 3 types of scan sequences, namely, 2D T2 weighted turbo-spin echo (T2* TSE) scans, 3D Gradient Echo (GRE) scans, and Ultra Short Echo 3D Cone (UTE) scans.

3.3.3.1 2D T2 star Turbo Spin Echo

The parameters of the 2D T2* TSE scan sequence were as follows: FoV (read) = 115mm, FoV (phase) = 28.6%, slice thickness = 0.9mm with 25 coronal slices and 40% phase

Figure 3.1: MRI Scanning Protocol Setup. The mouse coil was strapped to the table and elevated to be directly in iso-center, which was required by the UTE scans. The anesthetic delivery system was sent through the bore of the magnet.

oversampling, base resolution = 384 with 100% phase resolution, all of which yielded a voxel size equal to 0.3 x 0.3 x 0.9mm. 2 averages and 1 concatenation were employed in pursuit of an optimized signal to noise ratio (SNR), but kept the acquisition time of the scan to approximately 3 minutes 36 seconds. The TR and TE were 8000ms and 68ms respectively, and flip angle was 150 degrees. Other acquisition parameters were a turbo factor of 12, a readout bandwidth of 343 Hz/pixel with a fast mode RF pulse selected and a slice distance factor (gap) of 50% (0.45mm).

3.3.3.2 3D Gradient Echo

The parameters of the 3D GRE scan sequence were as follows: FoV (read) = 64mm, FoV (phase) = 100%, slice thickness = 0.5mm with 256 slices and 0% phase oversampling, base resolution = 128 with 100% phase resolution, all of which yielded a voxel size equal to 0.5 x 0.5

x 0.5mm. 4 averages and 1 concatenation were employed in pursuit of an optimized SNR, but kept the acquisition time of the scan to approximately 15 minutes 15 seconds. The TR and TE were 8.9ms and 4.80ms respectively, and flip angle was 25 degrees. Other acquisition parameters were, a readout bandwidth of 590 Hz/pixel with a fast mode RF pulse selected and a slice distance factor (gap) of 20% (0.45mm).

3.3.3.3 Ultra short Echo 3D Cones

The parameters for all four UTE scan sequences were the same with the exception of the TE times, which were; 0.3ms, 0.6ms, 1.2ms and 2.4ms. The scan parameters of the UTE scan sequences were as follows: FoV (read) = 120mm, FoV (phase) = 100%. The UTE scans used a protocol which generated 32840 3D cones. The parameters yielded a voxel size equal to 0.8 x 0.8 x 0.8mm. 1 average and 1 concatenation were employed in pursuit of an optimized SNR, but kept the acquisition time of the scan to approximately 6 minutes per TE sequence for a total of approximately 24 minutes. Other acquisition parameters were, a flip angle of 10 degrees, a readout bandwidth of 1560 Hz/pixel with a fast mode RF pulse selected. The UTE scans were reconstructed using a semi-automated reconstruction pipeline script developed at BYU (Provo, UT) by the Bangerter Group and their collaborators, run using MATLAB software (MathWorks, Natick, MA).

3.3.4 MRI Analysis: Estimation of Intensities

All MRI scans were analyzed using Horos software (Nimble Co LLC d/b/a Purview, Annapolis, MD USA) to calculate the signal intensities. An area of ~2.000 mm^2 (W: 1.694 mm H: 1.504 mm P: 5.0 mm) was chosen over bilateral hippocampi and used as our region of interest (ROI) (Figure 3.2). We aligned using the few visible landmarks such as the narrowing towards the frontal lobe, and the point where the brainstem and spinal cord descend in the neck. The mean signal intensities from ROI were normalized with the mean intensities from a water standard that

was included in each scan on the left side of the mouse. The water was used as an internal standard to control for the slight variation inherently present in every scan. The average mean values were analyzed on SPSS software.

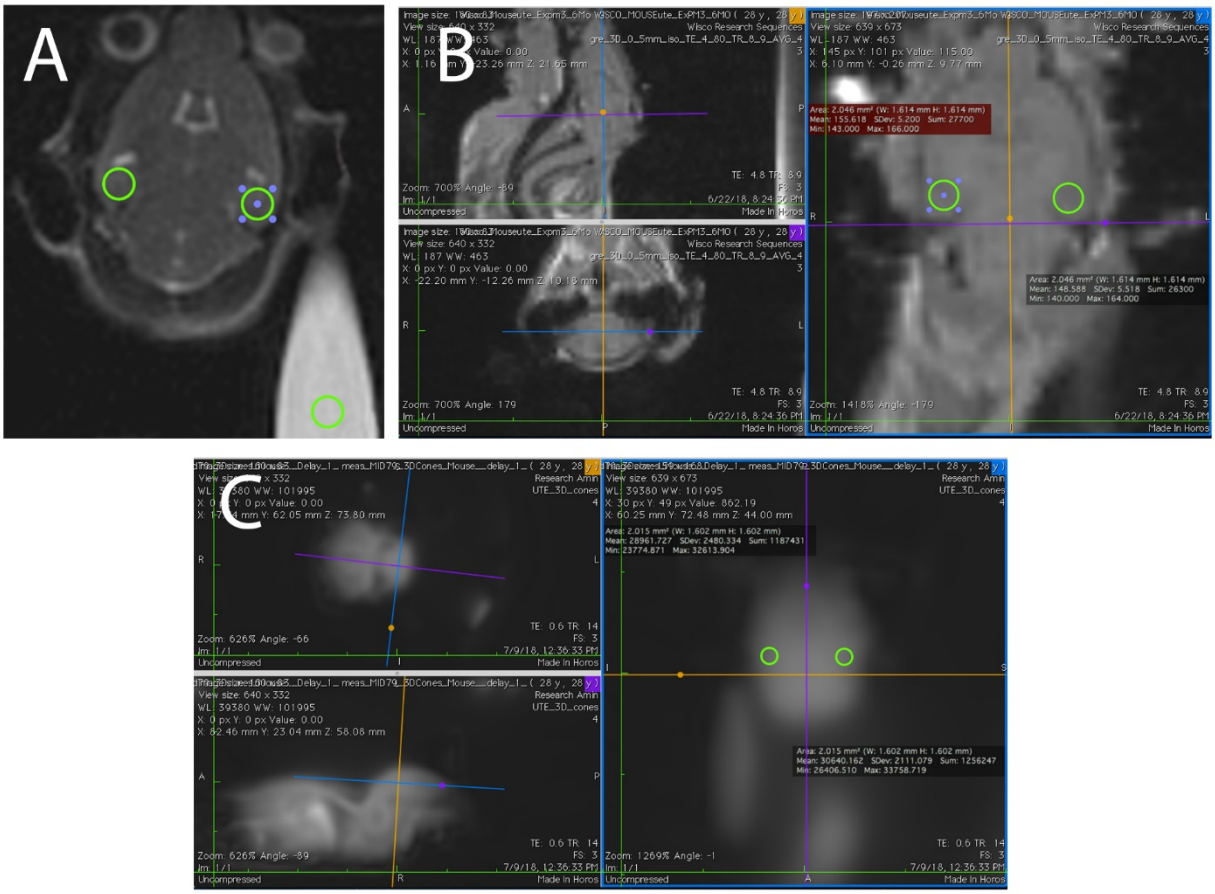

Figure 3.2: MRI Signal Intensities. ROI's with an area of 2mm squared were chosen over hippocampi (bilateral). And then normalized to a water standard included in each scan. (A) T2* TSE ROI on left and right hippocampus and the water standard in the bottom right hand corner. (B) GRE ROI over bilateral hippocampi. The crosshair pattern is noted and replicated in the following UTE scans. (C) UTE ROI example. Due to lack of resolution, the pattern for the GRE scan is replicated to zero in on the bilateral hippocampi. The water standard is easily discerned in all of the scans, and aids in orientation as the UTE sequence flips the orientation of the scan in relation to the GRE scan.

3.4 Results

3.4.1 MRI GRE and UTE Scans

Localizing at the left and right hippocampus, and standardizing to a vial of water included with each scan, we collected the mean intensities for the T2* TSE, 3D GRE, and 3D CONE UTE scans at TE values of .3 ms, .6 ms, 1.2 ms and 2.4 ms. There was a statistically significant

difference of intensity values based on the diet treatment administered, F (25, 5.217) = 6.984, p = 0.017; Wilk's Λ = 0.000; and a trend towards significance based on the genotype of the mouse, F(10,2) = 15.449, p = 0.062; Wilk's Λ = 0.000; and based on the sex of the mouse, F(5,1) = 83.179, p = 0.083; Wilk's Λ = 0.002; as well as a statistically significant interaction effect between diet treatment administered and genotype, F(30,6) = 5.341, p = 0.022; Wilk's Λ = 0.000; and between diet treatment administered and the sex of the mouse, F(20, 4.266) = 13.995, p = 0.008; Wilk's Λ = 0.000. The post hoc Tukey HSD multiple comparisons test revealed a significant difference between treatment group 1 and group 9 right hippocampus (-.297 +/- .066, p = 0.043) and 10 left (-.475 +/- .098, p = 0.030) and right (-.547 +/- .067, p = 0.003) hippocampus with the UTE 1.2ms scan.

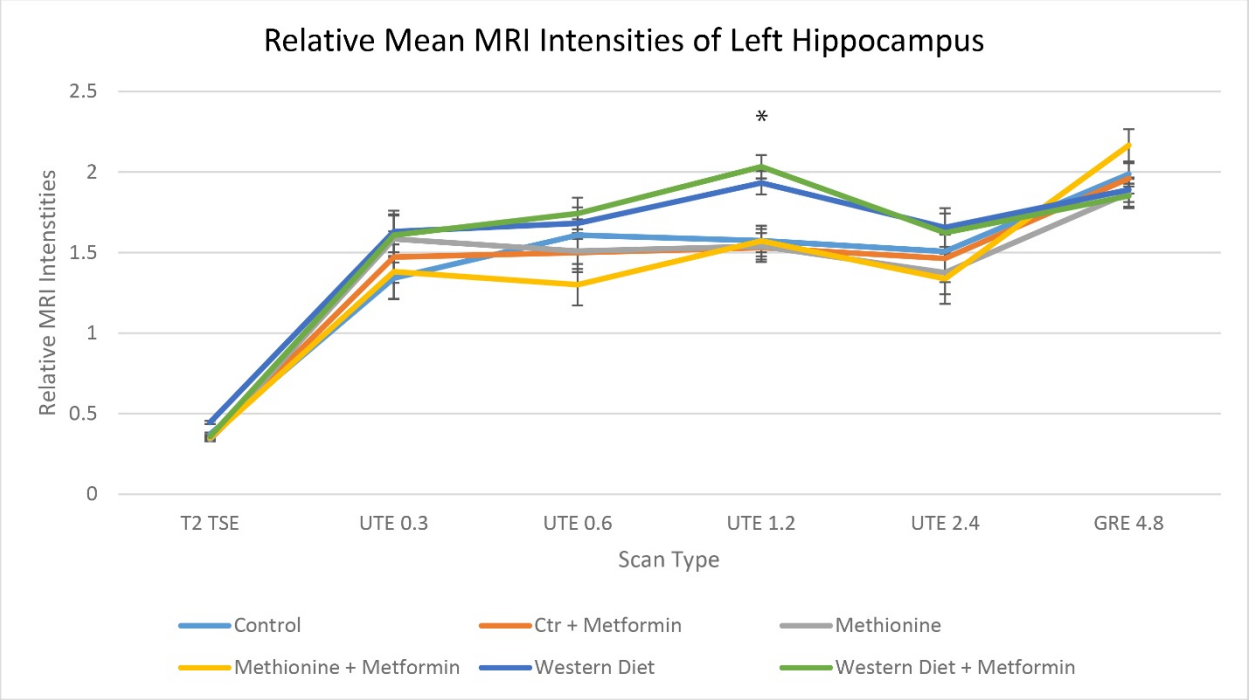

Figure 3.3: Relative Mean MRI Intensities of Left Hippocampus.

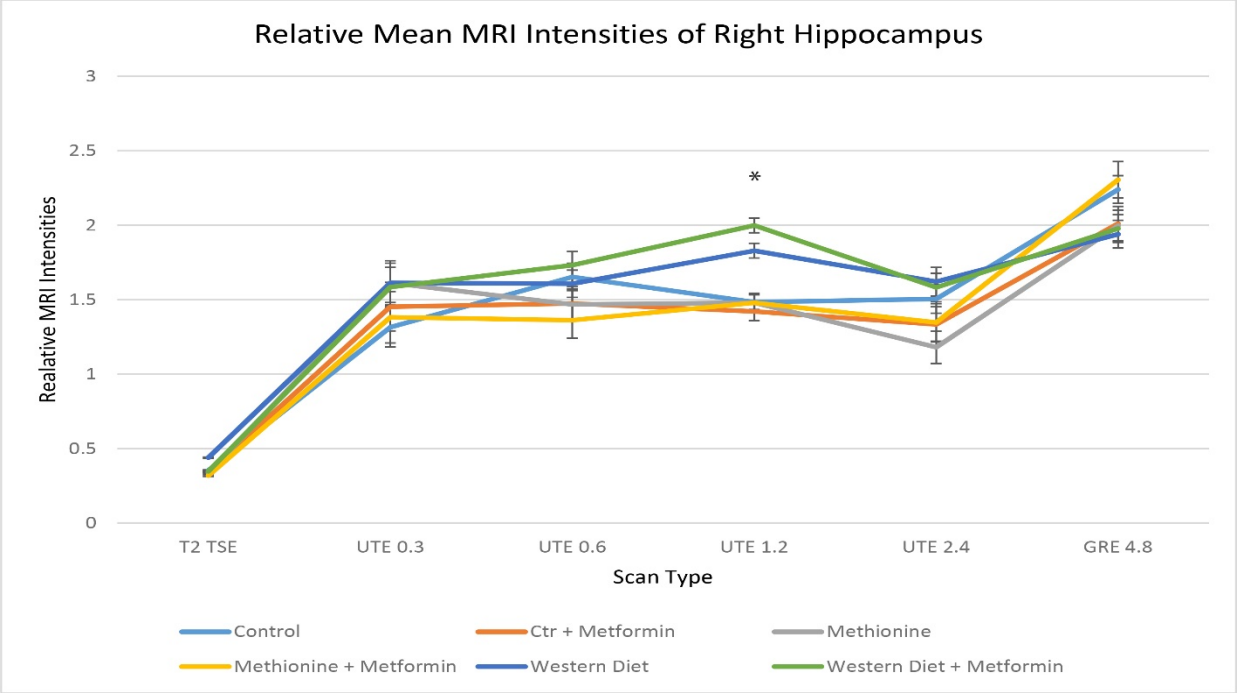

Figure 3.4: Relative Mean MRI Intensities of Right Hippocampus.

3.5 Discussions

We saw a wide range of mouse sizes and weights due to our diet treatments. The original protocol for scanning mice in the BYU MRI Research Facility called for the mouse to be placed within a 50 ml conical tube, which was then attached to a specially fabricated nose cone for optimum delivery of the isoflurane. This proved difficult as some of the mice became twice the size of an aged matched subject, some almost reaching 50 grams. It is also within this cohort of western diet treated mice that we saw the greatest significant difference. In Figure 3.3 and Figure 3.4 we see each of the diet treatment groups follows a uniform path from one scan to the other, except with the UTE 3D Cone sequence acquired with a TE of 1.2 ms. At this point we see an increase in relative signal intensity. For UTE scans, brighter signal indicates a higher concentration of iron bound proteins. In typical T2 weighted images we would expect a region with higher quantities of iron to have lower signal intensity as has been demonstrated previously with serial

concentrations of iron embedded within a phantom (Lu et al., 2018); therefore our results are consistent.

Given the signal intensity difference at 1.2 ms it is still possible that the increase in signal intensities is only seen with the western diet cohorts because it is due to an increase in fat. While UTE can very effectively visualize short T2 signals usually masked with conventional scanning sequences, in certain circumstances, especially in fatty or high water containing tissues, it becomes necessary to employ fat suppression techniques or long T2 nullification methods as shown by Nazaran et.al. and others (Carl, Bydder, & Du, 2016; Nazaran et al., 2017). It is also interesting to note that post processing of the images using the T2 signal acquired from the GRE scans may help to resolve and nullify disruptive long T2 signal still present in the UTE scans (Wang et al., 2014).

3.6 Conclusions

The scanning technique of 3D UTE Cones can be applied to murine and other small rodent models. There are significant differences present in the mean signal intensities of the hippocampus due to treatment with the western diet. This may be due to increased iron concentrations which are more easily visible using fat suppression techniques and nullification methods. The application of UTE will only improve with human subjects as there is more abundant signal present, assuming again, suppression and nullification techniques are applied.

3.7 Acknowledgements

The authors wish to thank the following funding sources: NIH/NIA 1 R21 AG037843; Brigham Young University, College of Life Sciences, Mentoring Environment Grant; Brigham Young University, School of Family Life, Gerontology Program; Brigham Young University, Magnetic Resonance Imaging Research Facility Seed Grant; Dr. Sarah M. McGinty Neuroscience Graduate Student Research Fellowship; Neurodar, LLC; Limitless Worldwide, LLC.

CHAPTER 4: General Conclusions and Importance

Alzheimer's disease (AD) rates are climbing every day, and while so many feel helpless against its impending arrival, we have the opportunity to chip ever so slightly away from the shell which masks the inner workings of the disease, and that is of the utmost importance. Using our transgenic model, we have shown the effect of those transgenes in the elevation of oxidative stress, and the potential in diagnosis using MRI, and treatment using established drug interventions.

It seems that we counted every step of the mice as we examined their behavior. Many undergraduates research associates grew in their scientific passion as we investigated the effect that diet has on both exacerbating and ameliorating the effects of oxidative stress and their impact on AD progression. We showed the efficacy of the transgenic mouse model in mimicking the effects of AD, and took it a step further to connect the neurodegenerative disease to another debilitating condition, diabetes. Our western diet treated mice performed more poorly in nearly every measure than their wild type control cohorts. Furthermore, we began to elucidate further the potential of metformin as a treatment for oxidative induced neurodegenerative diseases, with the added caveat, that it may have deleterious effects under normal physiological conditions.

Lastly, we made further steps in the journey to diagnose and predict the disease using the tool of MRI. Our method has great potential, and while we had significance in our measures, the diet treatments may have impacted our ability to measure what we had intended.

As with any journey, there really is no end, because the final steps of one journey are simply the initial steps of the next one. Using the tools and skills that one has learned to improve and move forward, one step at a time, is the real task and the real journey.

CHAPTER 5: Future Directions and Other Projects

5.1 Anatomy Academy

In the summer of 2012, while interning with Dr. Wisco at UCLA, I joined the first session of a brand new service learning program called Anatomy Academy. The program was developed by two medical students and an MPH/MPP student who saw a growing problem of childhood obesity and a gap in the preventative education at the 5-6th grade level. Their aim was simple: impact childhood obesity by teaching anatomy and physiology and nutrition in a didactic setting using premedical undergraduate volunteers as mentor teachers. Started as a summer program, Anatomy Academy saw great success in the first group of underserved students who expressed a greater interest in the material by the end of the week long program, and at the same time learned the material, evidenced by improved scores at the end of the week. This was the first time that I had ever taught anatomy and physiology to anyone, let alone children. It sparked in me, however, a flame for teaching that has never gone out. More importantly, I learned that teaching can be engaging and fun, with the same result as the less fun lecturing that I was used to.

I saw the benefit that the program had on the kids as well as myself and wanted to continue it, with Dr. Wisco's help and guidance, back at BYU. We translated the program into a 7 week course that ran concurrently with the normal Fall and Winter semester schedules. We quickly grew the program from just one session to dozens over the course of the next three semesters, plateauing at nearly 3 dozen sessions at 2 dozen schools, teaching over 800 elementary students using over 300 undergraduate pre-professional students. When I returned to BYU to begin my graduate degree, I knew that I would make Anatomy Academy a large part of my experience. Each semester I organized and trained the next groups of coordinators and mentors, helping the program to continue to expand and grow.

What started as just a program to help improve the lives of children, became much more than that for many of the undergraduate volunteers. Through reflections from the mentors, we saw that the ones who invested themselves into the program were often changed profoundly in their outlook of teaching, of service and of their own ability and goals. Some even changed career directions after having personal experiences that shook what they thought they knew and desired, and realizing a better path which aligned more with their goals and personalities. I had this same experience as I learned I had a passion for teaching and leading students to a better understanding of the material as well as its application to themselves. Anatomy Academy has taken me to two foreign countries, numerous conferences, presentations, workshops and posters in the 6 years that I have been involved. I have learned how to teach and how to lead, but most importantly I have learned how to learn and grow. I look forward to the next chapter of Anatomy Academy and what new lessons and challenges it will bring. Listed below are the poster abstracts and oral presentations in which I was involved for Anatomy Academy, which occurred at a variety of international conferences and meetings.

5.1.1 Poster Abstracts

Steed, K.S., Wisco J.J. Anatomy Academy: learning while serving or serving while learning? A narrative experience. American Association of Clinical Anatomists (ACAA) Regional Meeting Abstract 2017

Steed KS, Morton D, Wisco JJ. Anatomy Academy: A model for improving doctor-patient communication. International Association of Medical Science Educators (IAMSE). San Diego, CA 2015 Jun.

Steed, K.S., Wilson-Ashworth, H., Wisco, J.J. 2015. Anatomy Academy: A Catalyst For Personal Growth and Development In Undergraduate Pre-Professional and Medical Students. FASEB Journal 29, 1.

Steed, K.S., Diaz, M., Ojukwu, K., Padilla, J., Jenkins, K., Lassetter, J., Wisco, J.J., 2013. Our success in translating Anatomy Academy, an intervention program for 5th and 6th grade students, from Los Angeles, CA to Salt Lake City, UT. Faseb Journal 27, 1.

Banda, A.M., Steed, K., Schmalz, N., Ojukwu, K., Diaz, M., Padilla, J., Wisco, J.J., 2013. Effects of classroom structure on retention and implementation of knowledge in students. Faseb Journal 27, 1.

5.1.2 Presentations and Invited Talks

Steed K, Jensen R, Mulia M, White E, Wisco J. Anatomy Academy Model: Create Your Own Service-Learning Program. Human Anatomy and Physiology Society (HAPS). Salt Lake City UT. 2017 May.

Wisco JJ, Steed K, Lassetter J, Ray G, Morton D, Wilson-Ashworth HA, Stark ME, Seastrand G. A service-learning university-community partnership stewardship: The Anatomy Academy model. Center for the Improvement of Teacher Education & Schooling (CITES) Leaders Conference. January 16, 2015, St. George, UT.

Mageno A, Tullis A, Steed K, McCleve J, White E, Lassetter JH, Ray GL, Seastrand GE, Morton DA, Wilson-Ashworth H, et al. Who is the teacher and who is the student? The dual service- and engaged-learning pedagogical model in Anatomy Academy. Human Anatomy and Physiology Society (HAPS). Jacksonville FL. 2014 May.

Wisco JJ, Diaz M, Schmalz N, Steed KS, Jenkins K, Morton D, Lassetter JH. Anatomy Academy: A model program for exposing pre-professional and undergraduate medical and allied health sciences students to the six ACGME Core Competencies. International Association of Medical Science Educators (IAMSE). St. Andrews, Scotland. 2013 Jun.

5.1.3 Anatomy Academy Acknowledgements

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5.2 Teaching

What began as simply teaching elementary students blossomed into a passion for engaging students and helping them to learn how physiology (and other subjects) are can be exciting and applicable to each student in a personal way. With Dr. Wisco as a mentor, effective teaching and active learning have been a constant focus. Through m any opportunities of observing him teach, learning through special topic journal clubs and personal meeting I have already been made aware

of the many other options to the traditional lecture setting. While there is great wisdom in the traditional teaching pedagogy of Blooms Taxonomy, and recognizing a hierarchy of types of learning, I have always appreciated Dr. Wisco's approach of integrating each level, rather than waiting for mastery of one before attempting the next (Bloom, 1964).

5.2.1 Blooms Taxonomy

For example, Bloom teaches that lower levels of learning must be built, such as knowledge and comprehension, before higher levels, such as analysis and synthesis, can be attempted. While this is generally true, in real world application this is not how we go about learning. When learning how to ride a bike, does a child first have memorize (knowledge) every part of the bike, or the physics equations (comprehension) that keep them upright while pedaling, or the laws of friction and gravity that move the bike across the road, before hopping on the bike? No way, and if you tried to teach your kid how to ride a bike that way, they would lose interest pretty fast. Instead, we learn a basic foundational knowledge, but more importantly what that means to us personally e.g. this is the brake, it will help you stop when you are going to fast so you don't crash and hurt yourself. With some basic knowledge and comprehension, not mastery, a child will hop on the bike and start applying, which is the next level. Soon however, they will analyze their ability and quickly move into synthesis with tricks like no feet, no hands and the fated curb jump.

5.2.2 Fink's Taxonomy

The hierarchy dissolves into an amalgam of each level of learning occurring at the same time, because it matters to the individual, and educations changes into significant learning, which another educator, L. Dee Fink, outlines in a pedagogy called Fink's Taxonomy (Fink, 2003). The levels of learning become arranged as a Venn diagram, with more personal and human components added, all surrounding a goal of significant learning. This is the ultimate goal of every educator no

matter what pedagogy the follow, and why people get into educations; to help people grow, better themselves and ultimately better the world

5.2.3 Provo College

Armed with these pedagogies and a willingness to improve myself, I was given the opportunity of taking over a dual anatomy lecture and lab course, four or five weeks after it had already started during the 2015 fall semester. To complicate the situation more, despite my desire and drive, I had never taken an anatomy course during my undergraduate education, but was determined to become successful. Despite a rough start, I jumped right in and maintained a steady week lead of learning the material well enough to teach it to the students. Reflecting on the semester, I was forced to keep the lesson outlines nearly exactly the same, and thus missed out on significant learning activities, but overall it was a successful semester. Best of all, they invited me back to back to teach human physiology the following semester. With a bit more time to prepare, and a subject I was vastly more knowledgeable on, I set out to change as much as I could in the lectures to make them more engaging, peppered with activities, and as often as possible, discussion and reflection inducing. The semester was a great success, and I learned a lot about how to alter a curriculum gradually, and with purpose. I also learned that it is ok if activities don't work out exactly how you planned. The class size of Provo College was about 20 students, and was a nice and gentle first exposure to teaching at the college level. Unfortunately, because I did not have a finished degree, the administration, despite great reviews from students and faculty alike, decided a replacement was in order.

5.2.4 Utah Valley University

Coincidentally, after being let go from Provo College, a collaborator from Anatomy Academy, suggested I apply to Utah Valley University as a lab instructor. I opened an application

and was offered a position as a lecturer. I was given 3 sections with about 175 students total, and that was about all the instruction I was given. From the time I was hired, I didn't see an administrator until one showed up unannounced in my classroom a third of the way through the semester. While I appreciated the independence I was very surprised by how much I was given, but I used it to craft the course how I thought would work best with my students and my teaching style. Using some of the materials from Provo College that had worked well, combined with materials from my Anatomy Academy collaborator, we had a successful course, but there were two major issues with the semester. First, I was not used to the schedule I was given, and was constantly moving dates around to make the material match, which was a bit difficult for the students to follow along with their deadlines. Second, I had a difficult time writing my tests, and getting a good spread of grades, which was the biggest comment from the administrator after the semester. They suggested that I simply make the tests harder to spread the grades out, and curve back up to my desired average. I didn't like this option.

My second semester I decided that I would fairly increase the difficulty of the exams, by throwing out obvious questions, and replacing them with application questions. In order to compensate I stressed attendance of my office hours in the tutoring lab, which students actually attended and we covered more specific questions with deeper understanding. It was a success, the test scored spread out, but average stayed at around 74%. I likely still gave out too many A's for the administrations liking, but it was headed in the right direction, and overall the student's reactions were positive. In my teacher evaluations I was given an average of 4.6/5 for both of the semesters and only one major complaint. The complaint came from a student who I caught in a lie, because I removed points from assignments that I had extended past the due date for him. The administration investigated and sided with my decision, but he taught me to keep to the policies

that I establish at the beginning of the semester, and it will be better for me and the students in the long run. I absolutely loved teaching all my students, and if I had more time to finish my doctorate, I would have likely continued at UVU longer. I am looking forward to the next opportunity that I will have to teach at the college, and eventually even the medical level. Listed below are the educational oral presentations in which I was involved, which were at international conferences and meetings.

Wisco JJ, Read CC, Nguyen SE, Sanders LE, Dorius GT, Steed KS, Hutchinson B, Morton DA. The uncertainty principle of self-directed learning and a TA training program in response. American Association of Clinical Anatomists (AACA) Regional Meeting Abstr 2017

Wisco JJ, Steed KS, Morton D. Riddle Me This? Constructing Effective Multiple-Choice Questions. International Association of Medical Science Educators (IAMSE). Leiden, The Netherlands 2016 Jun.

5.3 Collaborations

I have learned of the many benefits afforded those who collaborate with other researchers and investigators. In my time at graduate school, I have had the opportunity to collaborate on several other projects with researchers from both within and without BYU. Collaboration is so important to me because I am not an expert in many of the fields surrounding Neuroscience, yet involved deeply with them despite that uncomfortable fact. As such, I rely on collaborations to help move my own projects forward, and it follows that when people come needing assistance, I feel obliged to help where I can. Many of the collaborations were with other graduate or undergraduate students who needed help with techniques with which I was already proficient. Several undergraduates needed help getting projects going that the MRI facility with either specimen scanning or animal scanning using the anesthetic machine. I collaborated with BreAnna Hutchinson, Bryan Crum and Jennifer Bowden, scanning hundreds of slides for them at an off-site lab histology slide scanner that I was trained to use during my first year of graduate school.

5.3.1 Larynx Hydration – University of Wisconsin, Madison

One project on which I spent many hours is a collaboration on a larynx and vocal fold hydration study with Renee King at the University of Wisconsin in Madison. We supplied readily available porcine larynges and ease of access to affordable MRI scan time, which turned out to be to very valuable resources for them. Renee and one of her lab associates came to Utah to perform the pilot study, and establish the experimental protocol, which I would continue after they returned to Madison. Over the course of the next several months I dissected, and treated 30 pig larynges with different dilutions of NaCl, PBS and pure water scanning each one at baseline, 30 minutes after dehydration and 30 minutes after rehydration with each PD weighted scan taking around 16 minutes including localization and prep. The following is the abstract from the manuscript submission we prepared and is currently under review at the time of this submission.

> Renee E King; Kevin Steed; Ana E. Rivera; Jonathan J Wisco; Susan L Thibeault. 2018. MRI quantification of dehydration and rehydration in vocal fold tissue layer. *PLoS One*
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> Clinicians commonly recommend increased hydration to patients with voice disorders. However, effects on clinical voice outcome measures have been inconsistent. Hydration-induced change within different layers of vocal fold tissue is currently unknown. Magnetic Resonance Imaging (MRI) is a promising method of noninvasively measuring water content in vocal folds. We sought to image and quantify changes in water content within vocal fold mucosa and thyroarytenoid muscle after dehydration and rehydration. Excised porcine larynges were imaged using proton density (PD) weighted MRI before and after immersion in hypertonic, isotonic, and hypotonic solutions. Larynges dehydrated in hypertonic solutions were rehydrated and imaged a third time. Scans revealed fluid-rich vocal fold mucosa that was distinct from muscle at baseline. Baseline normalized signal intensity in mucosa and muscle varied by left vs. right vocal fold ($p < 0.01$) and by anterior, middle, or posterior location ($p < 0.0001$). Intensity changes in the middle third of vocal fold mucosa differed by solution after immersion ($p < 0.01$). Hypertonic solutions dehydrated the middle third of mucosa by over 30% ($p < 0.001$). No difference from baseline was found in anterior or posterior mucosa or in muscle after immersion. No association was found between intensity change in mucosa and muscle after immersion. After rehydration, intensity did not differ by solution in any tissue, and was not different from baseline, but post-rehydration intensity was correlated with post-immersion intensity in both mucosa and muscle ($p < 0.05$), suggesting that degree of change in vocal fold water content induced by

hypertonic solutions ex vivo persists after rehydration. These results indicate that PD-MRI can be used to visualize large mammalian vocal fold tissue layers and to quantify changes in water content within vocal fold mucosa and thyroarytenoid muscle independently

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APPENDICES

Cell Culture Pilot Data: The Iron Hypothesis and its Effects on AD Associated Proteins in Cell Culture of Pyramidal Neurons

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Abstract

Alzheimer's disease has a multitude of established models to pursue when investigating its development and progression. While we had established a transgenic mouse model in general it made sense to start at the cellular level in our experiments. Dissecting out the hippocampi from neonatal pups from each of our cohorts, we plated the cells and applied our treatments directly into the mediums. The cells were cultured for 4 weeks, with stress treatments applied during weeks 2-4 and rescue treatments applied during weeks 3-4. We analyzed the lysed cellular contents using western blot for protein levels of various AD related proteins and a GSH/GSSG assay to detect levels of oxidative stress. Relative levels of AD associated proteins as run on western blot were not found to be statistically significant. There was a significant difference of the relative ratios of the reduced and oxidized forms of glutathione between genotype for the cells cultured. The results here show that further investigation is required for better elucidation of the processes involved.

Introduction

Alzheimer's disease (AD) has been shown through many studies to be a multifaceted, and convoluted condition involving many systems, pathways and signaling cascades. Additionally, the combination of genetics and environmental contributions further complicates our understanding of AD's pathogenesis and progression, and therefore prevents our ability to treat it. There is, however no doubt of the disease's cellular location and characteristics, which have been well documented for well over 100 years (STELZMA, 1995). While a variety of research models are important for further understanding AD, one essential route of investigation is on the cells directly. By experimenting on the cells most commonly associated with the disease, one bypasses the safety and protective mechanisms afforded by the body's immune and circulatory systems as well as the physical obstacle provided by the blood brain barrier. While the results of the study will not be

directly applicable to the physiological conditions of the disease seen in humans, the understanding provided by the cells reactions to direct treatments will help us to better understand the intricacies of the unruly disease.

Materials and Methods

Animal Husbandry

To supply the cells for our primary culture lines we maintained and bred a mouse colony of several different mouse strains. Ordered from Jackson Laboratories and approved for experimental use by the BYU Provo Institutional Animal Care and Use Committee (IACUC), we maintained three strains in the BYU Life Science Building (LSB) Vivarium for cell culture, namely; C57BL/6J (WT), B6.Cg-Mapttm1(GFP)Klt Tg(MAPT)8cPdav/J (TAU), and B6;129-Psen1tm1Mpm-Tg(APPSwe,tauP301L)-1Lfa/Mmjax (PSEN1). Each of the breeders was fed ad libitum both water and Teklad diet TD.8604 (Envigo, Madison, Wisconsin USA) supplied by the LSB vivarium. One male and one female were placed together in the male cage for 5 days and then separated back to their individual cages. At 21 days the female was monitored and pups were

Table 0.1: Mouse Pup Dissections. Learning the protocol for dissection, platting and maintaining the culture took several month and during that time tau and WT mice were available. There was also a period where several reagents went bad and ruined multiple WT and Tau culture plates. Lastly, PSEN1 breeders started producing fewer pups towards the end, so the cells ended up being spread between more wells.

Mouse Pup Dissections

Genotype	# of Pups	Successful Wells Cultured
PSEN1	27	56
Tau	43	95
WT	42	95

removed and prepped for dissection within 24 hours of discovery, with a total of 112 pups dissected (Table 0.1). All procedures involving animals were reviewed and approved by the BYU IACUC, ensuring that animals were treated humanely and in accordance to IACUC guidelines.

Cell Culture Dissection

Separated pups were euthanized in a laminar flow hood through approved BYU IACUC methods, and immediately confirmed by decapitation. The entire cell culture and dissection protocol was followed by Beaudoin et.al, and contact was made with the authors regarding

Table 0.2: Mediums for Cell Culture. The basic recipes and catalog for each of the mediums (dissection, plating, and maintenance) was taken from same protocol that the culturing steps were taken from. The various types of maintenance medium treatment concentrations were drawn from the literature as commonly used elevated amounts, or amounts that had been tested previously.

Mediums Table

Medium Name	Components	Final concentration	Catalog Number
Dissection Med.	10x Hanks Balanced Salt Solution	97.50%	Invitrogen 14185-052
	Sodium Pyruvate (100X)	1x	Sigma P2256
	D-Glucose (45%)	0.10%	Sigma G8769
	1M HEPES	10mM	Invitrogen 15630-080
	ddH2O	-	BYU
Plating Med.	MEM	86.55%	BYU ChemStores
	FBS	10%	BYU ChemStores
	D-Glucose (45%)	0.45%	Sigma G8769
	Sodium Pyruvate (100x)	1x	Sigma P2256
	GlutaMAX (100x)	1x	Invitrogen 35050-061
	Pen/Strep (100x)	1x	Invitrogen 15140-122
Maintainance Med. (MM-A)	Neurobasal Med.	96%	Invitrogen 21103-049
	B-27 Supplement	1x	Invitrogen 17504-044
	GlutaMAX (100x)	1x	Invitrogen 35050-061
	Pen/Strep (100x)	1x	Invitrogen 15140-122
MM-B	.6 M Metformin	50 µM	ALX-270-432-G005
MM-C	D-Glucose (45%)	50mM (25mM in MM)	Sigma G8769
MM-D	D-Glucose (45%)	50mM (25mM in MM)	Sigma G8769
	.6 M Metformin	50 µM	ALX-270-432-G005
MM-E	L-Methinoine 100 mM	100 µM	M5308-25G
MM-F	L-Methinoine 100 mM	100 µM	M5308-25G
	.6 M Metformin	50 µM	ALX-270-432-G005
MM-G	Insulin .6 mM	2 µM (.6 µM in MM)	Sigma I5500
MM-H	Insulin .6 mM	2 µM (.6 µM in MM)	Sigma I5500
	.6 M Metformin	50 µM	ALX-270-432-G005

questions about the protocol (Beaudoin et al., 2012). A tail snip was kept and frozen at 80 C for genotyping and the brain was immediately excised, being careful not to puncture the meninges or disturb the brain tissue, and placed in Dissection Medium (DM) (See Table 0.2 for complete list of mediums) in a 60mm dish. The dish was transferred to a dissection microscope where the cerebellum was removed and the hemispheres separated shown in Figure 0.1-A. The meninges

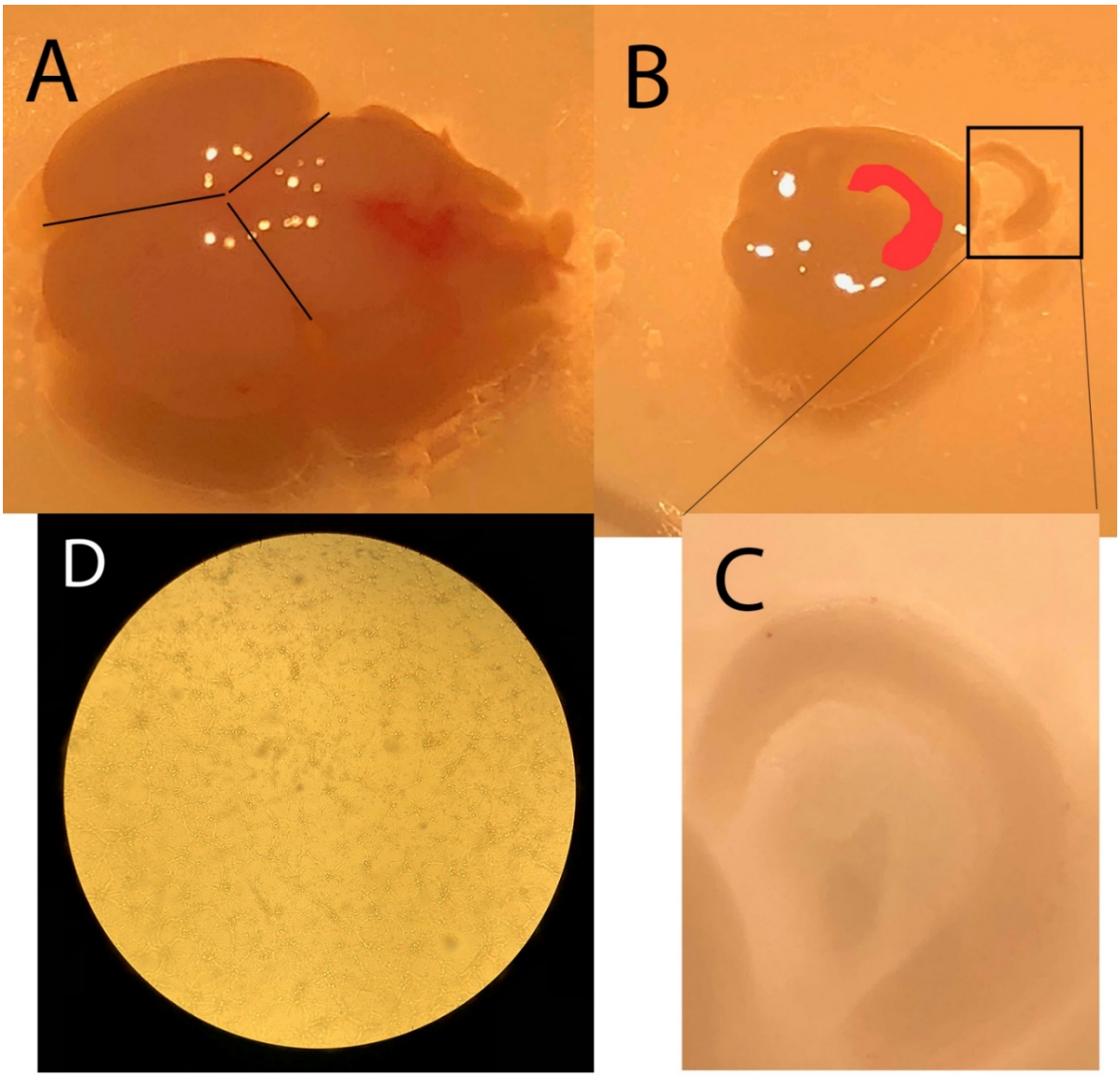

Figure 0.1: Dissection and Plating Procedure. (A)The mouse pup brain after being removed from the skull, with lines denoting where the cerebellum and hemispheres would be pinched to be separated. (B) A hemisphere laying on its lateral side with the excised hippocampus removed and boxed. The position of the hippocampus before removal has been shaded in red. (C) A close up view of the hippocampus with its distinctive curved shape and subtly darker color. (D) After dissection and processing of the tissue the cells are plated. The cells pictured are several weeks into development.

were then removed in order to reduce the chance of rapidly multiplying cells outgrowing the neuronal cells. Each hemisphere was then, in turn, rotated to sitting on its lateral side and the remaining thalamus and midbrain were removed revealing the hippocampus, which has a distinct curved shape and subtly darker appearance seen in Figure 0.1–B and C. Using forceps, the boundary of the hippocampal fold was pinched to release the entire structure, taking care to ensure that no meningeal fragments or vasculature remained on the surface or tucked behind. The left and right hippocampi were then combined in a 1.5ml micro-centrifuge tube with 1ml of dissection medium, and set aside for the disassociation steps and plating.

Cell Culture Plating

The process of cell plating began several days before the cells even touched the wells. The cells were adhered to a glass coverslip in a twelve well plate, but the coverslip was first etched using Nitric Acid, washed, sterilized and treated with poly-L-lysine to aid in cell adherence, all of which require overnight treatments. Once treated coverslips were prepped and placed in a 12 –well plate in maintenance medium (MM), they were stored in an incubator at 5% CO_2, 37 C and 100% humidity to await plating. The hippocampi from dissected pups were washed in fresh DM and incubated in 1.5% trypsin at 37 C for 20 minutes, followed by DNase incubation at RT for 5 minutes. After incubation the tissue is washed twice in DM and then washed twice in plating medium and brought to 500 µl before being triturated 20-30 times using a 1000µl micropipette to disassociate the cells. After the disassociation step a hemocytometer was used to acquire a cell count and 3-400,000 cells were plated in each well and the plate was returned to the incubator at the above specifications. After at least 24 hours of incubation the mixed medium of PM and MM was changed with fresh MM, leaving roughly half of the old medium, and therefore any of the excreted neurotrophic factors to aid in cell growth.

Cell Culture Maintenance and Treatments

Two times a week after plating, half of the medium was exchanged for fresh medium, which was also the mode of delivery for the treatments. As per the protocol, the cells were maintained for 28 days, with the first week allowing for cell adherence and growth (see Figure 0.1-D), the stress treatments during weeks 2-4 and rescue treatments during weeks 3-4. The precise molar concentrations of each of the treatments, found in Table 0.2, were added to individual preparations of the MM. All concentrations of drugs were confirmed to be relevant to either elevated physiological diseased levels, or published elevated research levels. All precautions possible were maintained to reduce the chance of contamination, including all work being performed in a hood, constant glove sanitization and hardware autoclaving, covering plates and medium bottles at all times. At the end of the fourth week of culture maintenance, the cells were harvested, lysed and contents stored at 80 C for future analysis.

Western Blot Analysis

The lysed cellular contents were run on 4-15% SDS-PAGE, and transferred to nitrocellulose paper for incubation with primary antibodies. Each of the membranes was cut into six pieces, above and below the target protein. We used the following AD associated primary antibodies to determine the relative quantities present in the mouse brains: APP, tau, ferritin, ferroportin, with GAPDH and Vinculin as a loading control. The presence of primary antibody was then visualized with a secondary antibody using LI-COR donkey-anti-rabbit IR-DYE (LI-COR Biosciences, Lincoln, NE) and images were taken using a LI-COR Odyssey CLx western blot scanner.

GSH/GSSG Measure of Oxidative Stress

Glutathione is a potent, endogenous antioxidant in our body, and as a result can be a great measure for the oxidative stress levels present in our cell cultures. The test looks at the ratio of the reduced and the oxidized forms of the antioxidant. We purchased a kit from Abcam (Cambridge, MA) and analyzed the samples and standards on a microplate reader at 485/528 nm.

Cell Culture Preliminary Results

Western Blot

We used the loading controls GAPDH and Vinculin to standardize the protein in each of the gels. Perhaps due to the low dilution of each of the cell lysates, there was increased variability within each sample, but assuming uniform amount of each loading control protein, based on its intensity we then standardized each of the accompanying target proteins. We needed multiple standards because our some of our target proteins were too close to either control with APP and Vinculin both above 100 kDa, and Ferritin/Tau and GAPDH all near the same range between 40 and 20 kDa. Preliminary results show a need for refining the quantification of protein for each lysate sample as well as increasing the number of samples run. Additionally, there were some interesting observations concerning the various proteins that were visualized on the gel, which will require further investigation and testing.

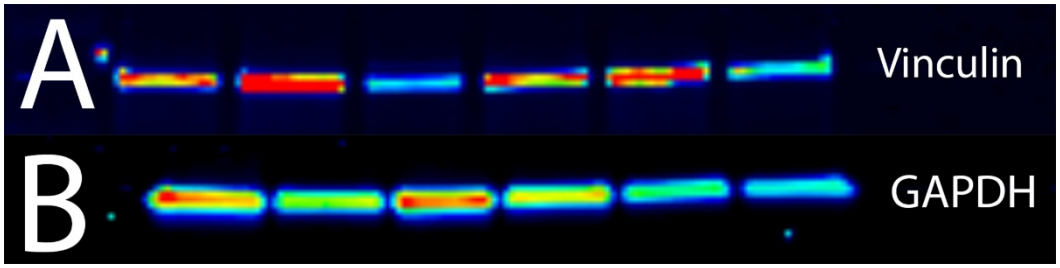

Figure 0.2: Western Blot Loading Controls. Each of the samples left to right are ordered: Tau, Psen1, WT of a stress treatment, and Tau, Psen1, WT of that same stress with rescue treatment. The heat map shows the relative intensities of each of the bands. (A) GAPDH loading control. (B) Vinculin loading control.

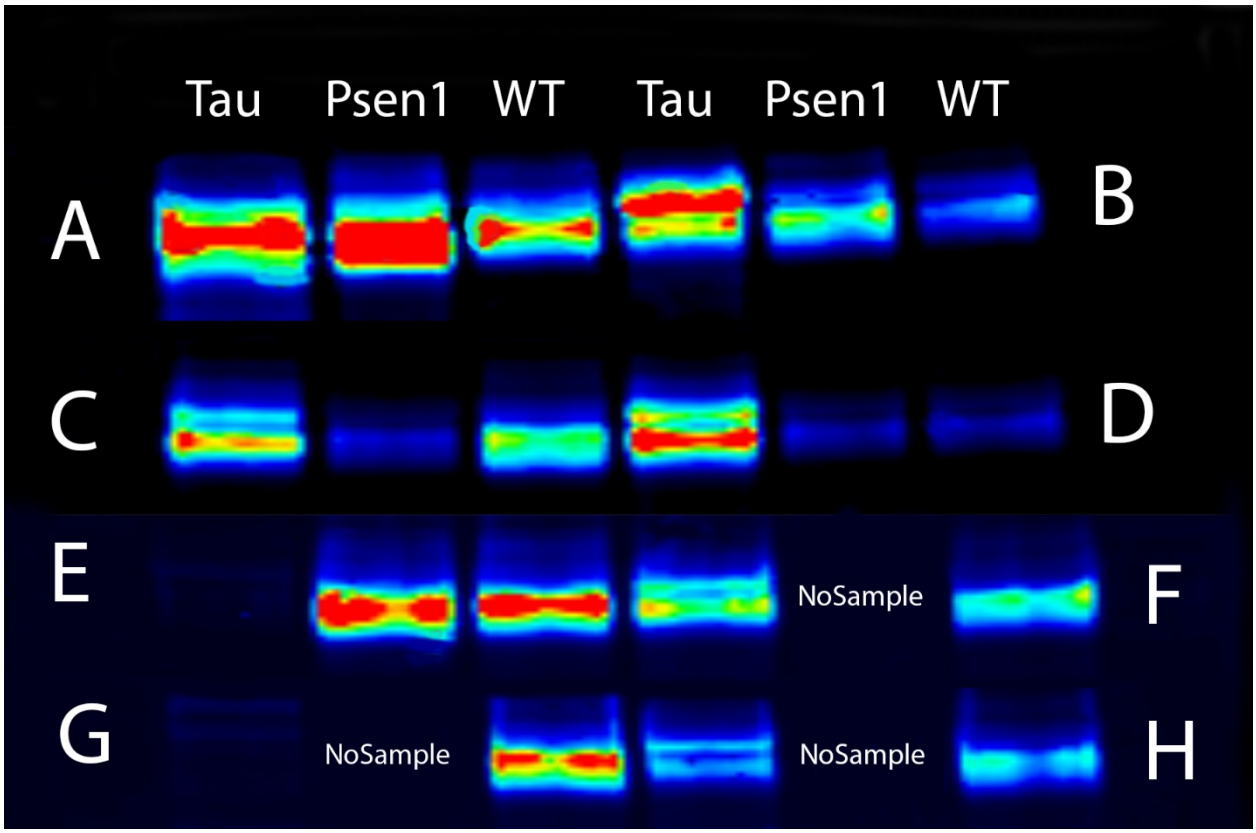

Figure 0.3: Tau Protein Western Blots. A combination of 4 different gels displaying the 8 different treatments applied to the different cell culture plates. The three band locations closest to a letter (A-H) belong to that treatment. E-Tau and G-Tau lack any visible tau protein despite both of their loading controls have sufficient intensity.

Tau Protein

The tau protein visualized with some of the greatest intensity overall of all the proteins besides the loading controls. Part of that is due to there being multiple isoforms of tau that can be visualized by the same antibody. The curious observation on our gels was in two separate tau transgenic mice, a complete lack of tau protein present. This occurred in two separate plates, whose cells were harvested from two completely different mice, although the mice came from the same mother (see Figure 0.3). We need to confirm the presence of the transgene, but even if the transgene was not passed on to the pup used to create those plates there should still be the endogenous tau protein as evidenced by sufficient signal showing up in the WT and PSEN1 mice, which carry no tau transgene. Treatment E was elevated levels of methionine and treatment G was elevated levels

of insulin, and both treatments were duplicated with rescue treatments added in F and H. Even more interesting is the fact that all other target proteins were still visualized, so it was not simply that the cells all died and the protein degraded. Further investigation will be sought in the future, as well as repeated western blots if there is sufficient lysate remaining. Additionally, it will be important to test another protein, such as NeuN or Beta-Tubulin, which are only found within neurons to ensure that neurons indeed were plated.

Glutathione Redox Assay

Initial results for the GSH/GSSG ratio assay are mixed and will require further analysis and refinement of the assay used. The ratios achieved are not in line with what we expected to see, especially considering the impact of the transgene that was seen in both behavior and anatomically with MRI and histology in other graduate dissertation work. We will need to repeat the experiment to ensure that our controls are consistent and examine the raw amounts of total glutathione between

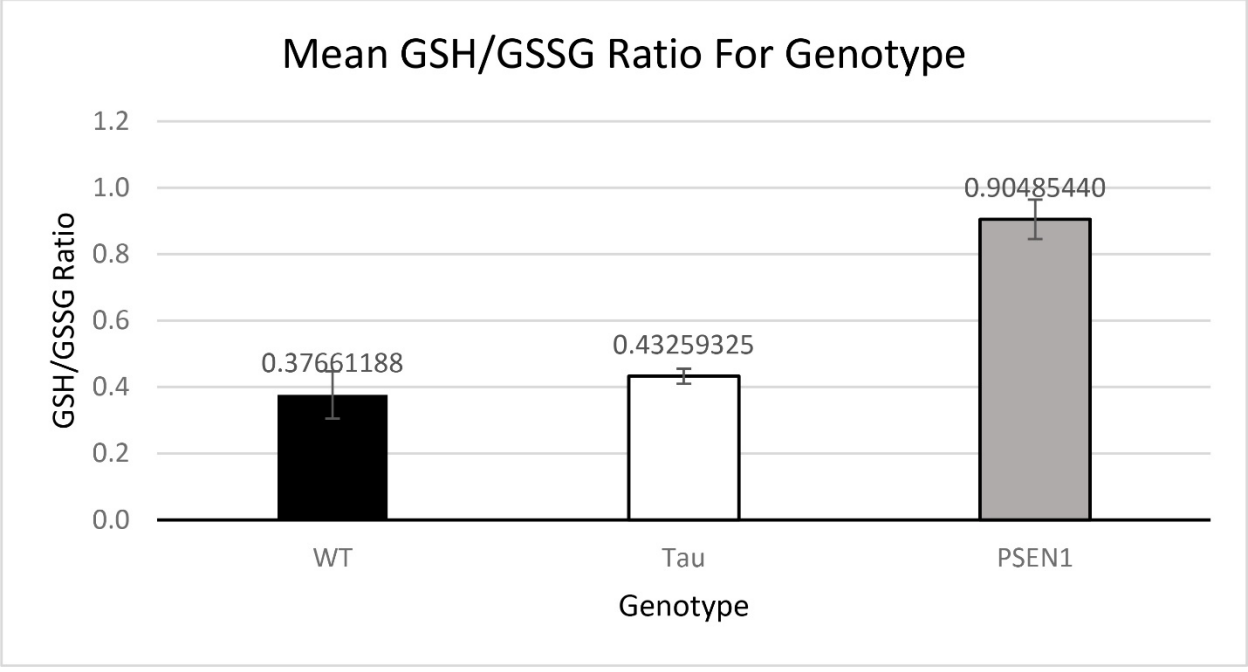

Figure 0.4: Mean GSH/GSSG Ratio for Genotype.

the samples as that may be a better indicator of the oxidative stress levels present within the cell cultures. Another pathway to consider is to confirm the results using another antioxidant assay.

Discussion

Western Blot Future Directions

In general, the results of the cell culture itself were very good. The cells were plated and grew for the 4 weeks as stated by the protocol. There was a learning curve to performing all the techniques correctly and optimizing the process for our needs. As with any experiment there were many technical difficulties and select cases of human error. Overall, however, there were very few wells that were lost because of experimental error, only 86 out of 346. Unfortunately, the amount of lysate collected from each well was only .578 µg/µl, and this was in only 100 µl of buffer or less. With that said, this was still adequate for most of our needs, but also meant that we had to be judicious with our use of the lysate in assays and experiments. It did make loading sufficient µg of sample in each well difficult as most of the lysates contained less than 10 µg in 15 µl, which was our maximum volume for the gels available to us. Despite all this trouble the western blots still need further refinement and a higher number of samples need to be tested. This is one area upon which we will focus in the future, optimizing and repeating the western blot protocol, as well as optimizing the cell culture and lysis protocol to increase the amount of lysate produced, and increase the concentration of protein within that lysate.

Glutathione Redox Assay

One of the measures that is important for the establishment of the iron hypothesis in an in-vivo model is for oxidative stress. When oxidative stress is elevated within the cell, there are certain endogenous molecules that will undergo redox reactions in order to prevent rampant redox reactions occurring between crucial enzymes, proteins or even DNA (Petrou, Petrou, Ntanos, &

Liapis, 2018). Among the most potent of these is glutathione, which as was mentioned previously is also important in the metabolic pathway involved with methionine and homocysteine. Glutathione exists most abundantly in its reduced form (GSH), but also exists in the oxidized form (GSSG) as well. When levels of oxidative stress increase GSH becomes GSSG to handle the stress (Zitka et al., 2012). If the state continues the normal ratio of roughly 100:1 GSH:GSSG will become skewed reaching closer to 10:1 or even 1:1. Our assay returned a significant difference between out PSEN1 at around 0.9, and WT and Tau respectively at .37 and .43. The ratio was calculated using a standard linear regression provided by the kit, which measured the reduced GSH in each sample and then the total Glutathione present in each sample. Taking the difference extrapolates the amount of GSSG present in the sample which is then used to get at GSH/GSSG ratio. Thus, WT and Tau conditions have higher levels of oxidative stress present on average, which could be explained by the fact that they each had 3 extra treatment groups included in their calculations. As mentioned earlier, the cell culture lysates were very dilute, and despite the cited pico-molar sensitivity of the assay, the results of that assay were all on the lower end of the standard curve. The results might be more reliable when we are able to repeat the assay, as well as run another form of oxidative stress/antioxidant assay independent of the GSH/GSSG assay.

Conclusions

Ultimately, the results at this point of the experiment were less successful than would have been hoped for. With the combination of time constraints, financial constraints and steep learning curves, the results need further refinement and additional testing. That does not, however, mean that it was a failed experience. The dissection of the pup brains worked well, and the cells were plated and grew consistently. Despite malfunctioning technologies best efforts, the experiments

moved forward, and we harvested and found that we had our target proteins. We plan to continue refining our method and answer the questions that arose from our findings.

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Education

Brigham Young University - PhD Neuroscience - Expected Graduation August 2018
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Research Experience

Research Assistant - August 2014 to Present
Dr. Jonathan Wisco, Physiology and Developmental Biology, BYU
Certification level 3 to operate MRI scanner - Assist in scans of human and animal tissue
Operate and maintain anesthetic machine for mouse imaging procedures
Manage a team of 30+ students in examining behavior and memory of mice in radial arm maze
Develop and maintain primary murine neuronal cell cultures used in experiments
Lead murine colony breeding and husbandry and confirm genotype of transgenic mice
Coordinate with other graduate students and their teams on overlapping projects

Research Assistant - August 2014 to December 2014
Dr. Jeffrey Edwards, Physiology and Developmental Biology, BYU
Run radial arm maze protocol
Certify to handle mice in experiments

Summer Intern Lab Fellow - June 2012 to August 2012
Dr. Jonathan Wisco, Pathology and Lab. Medicine, UCLA
Section brain tissue samples and subject them to a variety of staining protocols
Image stained tissue and ensure organization of hundreds of pictures using reconstruction programs

Summer Intern Lab Fellow - May 2011 to July 2011
Dr. Daniel Fowler, Transplantation Immunology, NIH
Western blot analysis of proteins from Th2 cells and analyze cytokine expression using flow cytometry
Culture cells and run cell killing assays for different drug ratios
Interpret results, plot data, and report to post-doctorate mentor daily and PI monthly

Research Assistant - January 2011 to January 2012
Dr. Laura Bridgewater, Department of Molecular Biology, BYU
Genotype mice to determine zygosity and sex and dissect osteoarthritic knockout mice for joint cartilage
Run western blot and expose on film for analysis

Dissect mice and stain skeletons for measuring and analyze for effects of gene knockout

Publications

Bridgewater, L.C., Mayo, J.L., Evanson, B.G., Whitt, M.E., Dean, S.A., Yates, J.D., Holden, D.N., Schmidt, A.D., Fox, C.L., Dhunghel, S., Steed, K.S., Adam, M.M., Nichols, C.A., Loganathan, S.K., Barrow, J.R., Hancock, C.R., 2013. A Novel Bone Morphogenetic Protein 2 Mutant Mouse, nBmp2NLS(tm), Displays Impaired Intracellular Ca2+ Handling in Skeletal Muscle. Biomed Research International, 11.

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Poster Abstracts

Steed, K.S., Wisco J.J. Anatomy Academy: learning while serving or serving while learning? A narrative experience. American Association of Clinical Anatomists (ACAA) Regional Meeting Abstract 2017

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Hansen, M.S., Salin, A., Barzee, B., Stone, M., Bridgewater, J., Kavafyan, T., Steed, K., Stark, E., Dong, H.W., Toga, A.W., Vinters, H.V., Wisco, J.J., 2013. Using imaging biomarkers in the histological validation of Alzheimer's disease. Faseb Journal 27, 1.

Barzee, B., Hansen, M., Salin, A., Stone, M., Bridgewater, J., Kavafyan, T., Steed, K., Stark, E., Dong, H.W., Toga, A.W., Vinters, H.V., Wisco, J.J., 2013. Histological validation of Alzheimer's disease and cerebrovascular disease imaging biomarkers. Faseb Journal 27, 1.

Salin, A., Hansen, M.S., Barzee, B., Stone, M., Bridgewater, J., Kavafyan, T., Steed, K., Stark, E., Dong, H.W., Toga, A., Vinters, H., Wisco, J., 2013. Histological validation of iron as an imaging biomarker for amyloid beta and tau depositions in Alzheimer's Disease. Faseb Journal 27, 1.

Presentations and Invited Talks

K.S. Steed, K.Barkdull, T. Hancock, J.J. Wisco. Effects of oxidative stress on transgenic mice: An Alzheimer's disease behavioral model. Program No. 357.08. 2017 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2017. Online.

Steed K, Jensen R, Mulia M, White E, Wisco J. Anatomy Academy Model: Create Your Own Service-Learning Program. Human Anatomy and Physiology Society (HAPS). Salt Lake City UT. 2017 May.

Wisco JJ, Read CC, Nguyen SE, Sanders LE, Dorius GT, Steed KS, Hutchinson B, Morton DA. The uncertainty principle of self-directed learning and a TA training program in response. American Association of Clinical Anatomists (AACA) Regional Meeting Abstr 2017

Wisco JJ, Steed KS, Morton D. Riddle Me This? Constructing Effective Multiple-Choice Questions. International Association of Medical Science Educators (IAMSE). Leiden, The Netherlands 2016 Jun.

Wisco JJ, Steed K, Lassetter J, Ray G, Morton D, Wilson-Ashworth HA, Stark ME, Seastrand G. A service-learning university-community partnership stewardship: The Anatomy Academy model. Center for the Improvement of Teacher Education & Schooling (CITES) Leaders Conference. January 16, 2015, St. George, UT.

Mageno A, Tullis A, Steed K, McCleve J, White E, Lassetter JH, Ray GL, Seastrand GE, Morton DA, Wilson-Ashworth H, et al. Who is the teacher and who is the student? The dual service- and engaged-learning pedagogical model in Anatomy Academy. Human Anatomy and Physiology Society (HAPS). Jacksonville FL. 2014 May.

Wisco JJ, Diaz M, Schmalz N, Steed KS, Jenkins K, Morton D, Lassetter JH. Anatomy Academy: A model program for exposing pre-professional and undergraduate medical and allied health sciences students to the six ACGME Core Competencies. International Association of Medical Science Educators (IAMSE). St. Andrews, Scotland. 2013 Jun.

Work and Volunteer Experience

Advanced Physiology Lab TA and Lecturer - August 2016 - December 2017
Brigham Young University, Provo, UT

Lead ½ of the lectures each semester, implementing active learning exercises and discussion
Guide 2-3 sections through the labs while fostering discussion and application of the topics
Restructure physiology lab curriculum learning objectives, labs activities, and assessments

Human Physiology Adjunct Professor - August 2016 - May 2017
Utah Valley University, Orem, UT
Hold reviews and office hours for students to address concerns in the classroom
Work with publishers and administration to build course curriculum
Develop active learning classroom activities to supplement lectures and course material

Anatomy and Physiology Adjunct Professor- October 2015 - May 2016
Provo College, Provo, UT
Lead didactic lecture and discussion on anatomy and physiology
Prepare lessons, learning activities and labs
Grade tests and hold office hours and reviews for students

Graduate Student Society Council Representative - August 2015 - Present
Brigham Young University, Provo, UT
Help organize and set up events for graduate students
Attend bi-monthly council meetings and plan events/provide feedback on previous events

Neuroscience Teaching Assistant - Jan - April 2015 and 2016
Brigham Young University, Provo, UT
Hold weekly review session and office hours to address lack of understanding and comprehension
Grade student quizzes and exams and provide feedback when necessary

Volunteer Mentor/ Head Coordinator - June 2012 to Present
UCLA/ BYU, Westwood, CA/ Provo, UT
Guide 5th & 6th graders through Anatomy Academy, setting goals for long-term healthy living
Receive and process applications for mentors, coordinate implementation of curriculum at new sites
Organize training of 250-300+ volunteers and help with IRB approval of curriculum changes

Donor Processor/ Lead Phlebotomist and Trainer - November 2011 to August 2014
Biomat USA: Grifols, Provo, UT/ Mesa, AZ
Document accurately, concurrently and consistently all things that occur with each donor
Screen donors by performing finger stick, taking vitals, and asking medical history questions
Perform aseptic venipuncture of donors and monitor all donors throughout donation process

Operate aphaeresis machine, know error codes and protocols for every situation and clean machines
Lead team of phlebotomists by setting goals, accountability and training new phlebotomists

Lead Student/Warehouse Worker - August 2009 to April 2011
Brigham Young University, Provo, UT
Receive new products, ensure accurate delivery, delegate tasks to other students
Pull orders for various departments and cafeterias on campus and deliver them efficiently
Deliver orders throughout campus, restock shelves, and maintain general upkeep of the warehouse

Carpenter - March 2009 to June 2009 and May 2012 to August 2012
Residential Renovations, Sacramento, CA and Barton Construction, Los Angeles, CA
Demolition, planning, and construction according to code and clients' specifications
Finish work, including framing, painting, electrical, plumbing, drywall, and appliance installation

LDS Missionary Volunteer - February 2007 to February 2009
Belgium Brussels/ Netherlands Mission
Led 30-40 full time volunteers in daily goal setting and reporting over several month periods
Learned to speak and write Dutch fluently as well as understanding Dutch culture and customs
Utilized team and partner teaching styles to effectively teach new material

Awards and Achievements

IAMSE Travel Award - June 2016
Leiden, the Netherlands
BYU Grad Expo 1st Place - April 2016, April 2017
Provo, UT
BYU Grad Expo Honorable Mention - March 2015, April 2018
Provo, UT
BYU Research Presentation Award - March 2015, October 2017
Provo, UT
American Association of Anatomists Travel Award - March 2015, April 2013
Boston, MA
BYU MRI Research Facility Level 3 Technician - January 2015
Provo, UT
Lolo Longboards - June 2006 to Present
Placerville, CA
Design, craft and sell custom longboards
Eagle Scout Award - August 2005
Placerville, CA
Renovated my elementary playground (benches, wall-ball courts and school sign)
Societies and Affiliations

American Association of Anatomists - 2013-Present
International Association of Medical Science Educators - 2015-Present
Society for Neuroscience - 2017-Present

Undergraduate Lab Associates

Spring/ Summer 2018

Jonathan Loveloand	Any-Maze Analysis
Jaythan Cunico	RAM/ Any-Maze Analysis
Julia Huff	MRI Assistant
Jackson Call	RAM
Kevin Noorda	RAM/ MRI Assistant
Keith Noorda	RAM/ MRI Assistant
Shane Stapleton	RAM/ Any-Maze Analysis
Matthew Harris	Any-Maze Analysis
Trey Novak	RAM
Ethan Ouzts	RAM
Truman Davidson	RAM
Jackson Pingree	RAM
Stuart Meyers	RAM
Tyler Loutensock	RAM
David Delgado	RAM
Katherine Valencia	RAM/ MRI Assistant
Megan Stephani	Any-Maze Analysis Lead
Chace Davies	Any-Maze Analysis
Ella Hale	Any-Maze Analysis
Owen Carter	Any-Maze Analysis

Winter 2018

Alex Wilkinson	RAM/ Any-Maze Analysis
Ikenna Nwosu	Cell Culture/ Any-Maze Analysis
Matthew Harris	Any-Maze Analysis Lead
Kevin Noorda	RAM/ MRI Assistant
Keith Noorda	RAM/ MRI Assistant
Cassie Childs	Cell Culture/ Any-Maze Analysis
Jonathan Loveloand	RAM/ Any-Maze Analysis
Ana Vasquez	RAM/ Any-Maze Analysis
Chase Westerlind	RAM
Hannah Brown	RAM
Tiffany Ferrel	RAM
Jessica Healy	RAM
Mattie Myers	RAM
Jackson Call	RAM
Natalie Stepheson	RAM
Da Young Jess Yoon	RAM
Julia Huff	RAM
Nicole Issac	Any-Maze Analysis
Shane Stapleton	RAM/ Any-Maze Analysis
Jaythan Cunico	RAM/ Any-Maze Analysis

Chace Davies	Any-Maze Analysis

Fall 2017

Alex Wilkinson	Any-Maze Analysis
Cody Buhler	Any-Maze Analysis
Ikenna Nwosu	Cell Culture/ Any-Maze Analysis
Matthew Harris	Any-Maze Analysis Lead
Bryant Rmafjord	Cell Culture/ Any-Maze Analysis
Kevin Noorda	Any-Maze Analysis
Keith Noorda	Any-Maze Analysis
Cassie Childs	Any-Maze Analysis
Jonathan Loveloand	Any-Maze Analysis
Ana Vasquez	Any-Maze Analysis

All Other Previous Semesters

Alex Wilkinson	RAM/ Any-Maze Analysis
Cody Buhler	RAM/ Any-Maze Analysis
Ikenna Nwosu	Any-Maze Analysis
Matthew Harris	Any-Maze Analysis
Paul Leavitt	RAM/ Any-Maze Analysis
Raquel Hoopes	RAM/ Any-Maze Analysis
Kaitlin Williams	Any-Maze Analysis
Rebecca Huang	RAM/ Any-Maze Analysis
Abbey Rasch	Any-Maze Analysis
Richard Lee	RAM/ Any-Maze Analysis
Kyle Barkdull	RAM/ Any-Maze Analysis
Bryant Rmafjord	Any-Maze Analysis
Claire Thomas	RAM/ Any-Maze Analysis
Hiva Lee	RAM/ Any-Maze Analysis
Julia Doh	RAM/ Any-Maze Analysis
Michael Swainston	RAM/ Any-Maze Analysis
Forrest Hamrick	RAM/ Any-Maze Analysis
Aubree Stoddard	RAM/ Any-Maze Analysis
Carine Johnson	RAM/ Any-Maze Analysis
Carlee Larsen	RAM/ Any-Maze Analysis
Colin Montgomery	RAM/ Any-Maze Analysis
Daxton Johnson	RAM/ Any-Maze Analysis
Elizabeth Cates	RAM/ Any-Maze Analysis
Mackenzie Anderson	RAM/ Any-Maze Analysis
Michelle Hamson	RAM/ Any-Maze Analysis
Miguel Vanegas	RAM/ Any-Maze Analysis
Rebecca Huang	RAM/ Any-Maze Analysis
Ryan Staudte	RAM/ Any-Maze Analysis
Samuel Young	RAM/ Any-Maze Analysis
Adam Holbrook	RAM/ Any-Maze Analysis
Alex Dorrough	RAM/ Any-Maze Analysis

Darius Rose	RAM/ Any-Maze Analysis
Hayden Doughty	RAM/ Any-Maze Analysis
Jeanie Kang	RAM/ Any-Maze Analysis
Jeff Stephens	RAM/ Any-Maze Analysis
Lelann Latu	RAM/ Any-Maze Analysis
Megan Anderson	RAM/ Any-Maze Analysis
Mika Honda	RAM/ Any-Maze Analysis
Shawn White	RAM/ Any-Maze Analysis
Taylor Leavitt	RAM/ Any-Maze Analysis
Ty Weisenburger	RAM/ Any-Maze Analysis
Tyler Boyack	RAM/ Any-Maze Analysis
Vince Labinpuno	RAM/ Any-Maze Analysis
Wen Xia	RAM/ Any-Maze Analysis
Zachary Motto	RAM/ Any-Maze Analysis
Devin Morris	RAM/ Any-Maze Analysis
Kayla Adams	RAM/ Any-Maze Analysis
Kjerstie Olson	RAM/ Any-Maze Analysis
Chandler M. Cottam	RAM/ Any-Maze Analysis
Natasha Harris	Any-Maze Analysis
Ethan Bryce	RAM/ Any-Maze Analysis
Lauren Manwaring	RAM/ Any-Maze Analysis
Taylor Leavitt	RAM/ Any-Maze Analysis
Scott Sonne	RAM/ Any-Maze Analysis