THE ROLE OF DIETARY COPPER AND ZINC ON INFLAMMATION AND OXIDATIVE STRESS IN ALZHEIMER’S DISEASE

by

Katelyn Boggs
A Thesis
Submitted to the Graduate Faculty of George Mason University in Partial Fulfillment of The Requirements for the Degree of Master of Arts Psychology

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___________________________________________ Department Chairperson

___________________________________________ Dean, College of Humanities and Social Sciences

Date: __________________________ Fall Semester 2015
George Mason University
Fairfax, VA
The Role of Dietary Copper and Zinc on Inflammation and Oxidative Stress in Alzheimer’s Disease

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts Psychology at George Mason University

by

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Bachelor of Science
George Mason University, 2012

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Fall Semester 2015
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Fairfax, VA
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DEDICATION

This thesis is dedicated to my entire family, whose encouragement and support (both emotional and financial) have made graduate school possible.
ACKNOWLEDGEMENTS

I would like to thank the many friends, relatives, and supporters who have made this happen, especially my parents. I would also like to thank my research assistants, Johanna Mingos and Sophia Stavrou, who were of invaluable help on this project. Thanks also go out to my committee members, Dr. Jane Flinn, Dr. Charles Madden, and Dr. Jennifer Brielmaier, who have provided support and guidance during this project. Finally, thank you to Stephen Lippi, Justin King, and Dr. Dan Cox, who were always willing to help with any troubleshooting that arose.
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<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid Beta</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>CC</td>
<td>Copper Control</td>
</tr>
<tr>
<td>Cu-</td>
<td>Copper Deficient</td>
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<tr>
<td>hAPP</td>
<td>Human Amyloid Precursor Protein</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary Tangles</td>
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<td>PS1</td>
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<td>PS2</td>
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<tr>
<td>SOD-1</td>
<td>Superoxide Dismutase-1</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
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<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>Zn+</td>
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ABSTRACT

THE ROLE OF DIETARY COPPER AND ZINC ON INFLAMMATION AND OXIDATIVE STRESS IN ALZHEIMER’S DISEASE

Katelyn Boggs, M.A.

George Mason University, 2015

Thesis Director: Dr. Jane Flinn

The mechanisms underlying the pathogenesis of Alzheimer’s disease (AD) have long been studied, yet they remain poorly understood. Recent developments in the field of AD research established a role for trace metal homeostasis in AD pathogenesis. Additionally, inflammatory signaling pathways have been implicated in AD, and many of these pathways contain metal binding proteins. The present study will build on previous work in our lab, and focus on the role of dietary zinc and copper, as well as brain inflammation and oxidative stress, in an early onset model of AD. Previous work in our lab has found that excess dietary zinc can lead to copper deficiency and cause behavioral impairments in mice and rats. We have also found that increased dietary zinc leads to increased levels of pro-inflammatory cytokines IL-1β, and TNF-α in an AD mouse model. This study used wildtype (WT) control, and transgenic (Tg) mice expressing one copy of the human amyloid precursor protein mutation (hAPP), to examine the relationship between dietary zinc and copper on brain inflammation and oxidative stress.
in AD. Dietary manipulations included: copper deficient diet + lab water (Cu- animals), zinc enhanced water + copper control diet (Zn+ animals), and copper control diet + lab water (CC animals). To measure inflammation this study examined Tumor Necrosis Factor-alpha (TNF-α), a proinflammatory cytokine thought to be a key initiator/regulator of the immune response. This study also examined levels of Superoxide Dismutase 1 (SOD-1), to gain insight into the role of oxidative stress in AD, as SOD- is delicately maintained by zinc and copper levels. All biomarkers were evaluated by Western blot.
CHAPTER ONE: INTRODUCTION

Normal Aging versus Dementia

The effects of aging vary by individual depending on several factors including lifestyle, sex, and genetics. As the body ages, the brain ages as well, changing physically in size and vasculature (Peters, 2006), with an accompanying decline in cognition as a result of these physical changes. One study reported a 5% rate of decrease in brain volume per decade after age 40, with the rate increasing after age 70 (Peters, 2006). The most common cognitive change observed with age is memory loss, a normal consequence of growing older. However, when one begins to experience impairments in activities of daily living as a consequence of memory loss, the cause is likely pathological. Dementia itself is non-specific; it is an umbrella term used to describe progressive cognitive impairment that results in the inability to perform everyday activities, ultimately robbing patients of their autonomy. There are many causes of the pathological changes observed with dementia, the most common being Alzheimer’s disease (AD), which is characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs).

Genetic Risk Factors and APP

Extracellular amyloid plaque formation is triggered by the abnormal accumulation of amyloid beta (Aβ) peptide, derived from a larger integral membrane protein known as amyloid precursor protein (APP). Processing of APP is done by three protein cleaving
enzymes: α-secretase, β-secretase, and γ-secretase. When processed appropriately, APP is cleaved first by α-secretase resulting in a soluble extracellular fragment that is actually thought to be neuroprotective, and a cell membrane bound fragment, which gets further cleaved by γ-secretase. After cleavage by γ-secretase, another soluble extracellular fragment is produced, and again a cell membrane bound fragment is left at the cell surface where it plays a physiological role in cell signaling; this is referred to as the non-amyloidogenic pathway (Vasto, Candore, Listi, Balistreri, Malavolta & Lio, 2008).

In the amyloidogenic pathway, abnormal processing of APP occurs when it is cleaved first by β-secretase, at a different location than cleavage by α-secretase, resulting in a smaller soluble extracellular fragment and a larger cell membrane bound fragment (McDowall 2006). This membrane bound fragment is then further cleaved by γ-secretase, resulting in a much larger extracellular fragment known as Aβ peptide. (Vasto et al. 2008). This resulting Aβ peptide is much longer than other fragments produced by non-pathogenic APP processing, which influences its aggregation into neurotoxic, insoluble plaques.
Autosomal dominant mutations on APP, presenilin-1 (PS-1), and presinilin-2 (PS-2) genes are heritable and account for most early onset AD (<65 years) cases by increasing the rate of pathogenic Aβ production (Zhang, Thompson, Zhang & Xu, 2011). Both PS-1 and PS-2 are transmembrane proteins and are cofactors in the processing of APP by the aforementioned secretases. Although there have been extensive studies on the physiological function of APP, its purpose remains unclear. The most consistently established function of APP is its role as a trophic factor, shown to stimulate neurite outgrowth (Thinakaran & Koo, 2008). Amyloid precursor protein has also been suggested to promote cell adhesion and act as a receptor (Kang et al., 1987)(Soba et al, 2005), though evidence for the latter is lacking. Murine studies have shown multiple times that modifications and deletions of APP have serious pathophysiological
repercussions. Mice lacking one of the genes encoding APP were lighter in body mass and experienced weakness in their extremities (Zheng et al., 1995). Further, these APP deficient mice showed deficits in learning, memory, and synaptic plasticity (Dawson et al., 1999). Certain double knockout APP mutations show early postnatal lethality, indicating that members of the APP gene family may have essential, overlapping functions. Deficiency of all three genes encoding APP leads to death shortly after birth (Thinakaran & Koo, 2008).

Copper/Zinc Balance

Many researchers have shown that Aβ plaques contain zinc, copper, and iron, all of which are essential trace metals. Zinc is the second most abundant trace metal in all eukaryotic organisms, and is required for many physiological processes such as protein folding, enzyme function, and replication (Coleman, 1992). Interestingly, APP expression is tightly regulated by zinc containing transcription factors, and itself contains a zinc-binding site in its promoter region (Vostrov & Quitschke, 1997). Zinc is absorbed by the small intestine after being consumed from the diet. The majority of zinc in the American diet is provided by red meat and poultry, though it is found in other foods such as beans, seafood, and nuts. In North America zinc deficiency is uncommon, but can be caused by vegetarianism, gastrointestinal disease, lactating women, sickle cell disease, and alcoholism (National Institutes of Health: Office of Dietary Supplements). Excess zinc in the diet is far more common than zinc deficiency, and consuming excess zinc is easier than most people are aware, as many foods are fortified with zinc. Zinc is also found in everyday items such as cough medicines, lozenges, denture creams, and nasal sprays.
Zinc toxicity can occur in both acute and chronic forms, and is linked to altered iron function, reduced immune function, and copper deficiency (National Institutes of Health: Office of Dietary Supplements). Copper deficiency as a result of excess zinc can occur because of zinc's ability to reduce the amount of copper absorbed by the small intestine (Maret & Sandstead, 2006). For this reason, it is recommended that copper supplements be taken with zinc supplements to reduce the possibility of developing zinc induced copper deficiency (Osredkar & Sustar, 2011).

There are three forms of zinc found in the brain:

1. Protein bound zinc, which is zinc that is bound to a protein.
2. Cytosolic free zinc, which is not bound to proteins and is available for use as a neuromodulator.
3. Vesicular zinc, which makes up only 10% of total brain zinc and is co-localized with glutamate.

Vesicular zinc has been shown to modulate synaptic transmission and plasticity by affecting the activity of both GABA and NMDA receptors thereby influencing inhibitory and excitatory transmission in the brain (Toth, 2011). Cells do not tolerate large fluctuations of homeostatic zinc levels, and if vesicular zinc is not immediately sequestered after being released into the synapse, it has the potential to induce beta amyloid aggregation through specific zinc binding sites on the Aβ peptide (Watt, Whitehouse & Hooper, 2010). Furthermore, brain regions with the highest levels of vesicular zinc include those involved in memory: hippocampus, amygdala, and cortex (Sindreu & Storm, 2011).
In addition to zinc, copper is also of physiological importance. Not only is copper found in amyloid plaques, there is often deficiency of copper in neighboring cells (Cater et al., 2008). Copper has the ability to modulate APP transcription, and APP protein processing, by switching APP breakdown from α-secretase (non-amyloidogenic) cleavage to β-secretase (amyloidogenic) cleavage. Under normal physiological conditions, copper is especially important for stabilizing superoxide dismutase, and maintaining the hosts natural anti-oxidant defense system (Cater et al., 2008).

**Oxidative Stress**

While zinc is thought to act as a neuromodulator in synaptic transmission, zinc also plays a role in mitochondrial trafficking. Under normal conditions mitochondria are incredibly mobile. They constantly move, divide, and fuse throughout the cell, positioning themselves to deliver ATP to regions of high energy demand, particularly near the synapse (Malaiyandi, Honick, Rintoul, Wang & Reynolds, 2005). Zinc is sequestered in mitochondria through the activation of a cation permeable channel, the mitochondrial Ca2+ uniporter, and its uptake by mitochondria provides clearance of cytosolic zinc accumulation (Sensi, Paoletti, Bush, & Sekler, 2009). Because mitochondria have the ability to perform transcription and translation, both of which involve zinc (Dineley, Votyakova & Reynolds, 2003), uptake of zinc by mitochondria is very sensitive and interruptions to its regulation, such as those caused by AD, can cause neurotoxicity.

When zinc begins accumulating in mitochondria, the mitochondrial membrane potential decreases and there is an increase in the production of reactive oxygen species
(ROS). Reactive oxygen species, also referred to as free radicals, are generated by oxygen and nitrogen based molecules that have unpaired electrons, making them unstable and highly reactive (Halliwell & Cross, 1994). In order to make paired electrons, they steal electrons from other molecules. Reactive oxygen species in the body are toxic if they aren’t neutralized, resulting in the ability to react with lipids, proteins, and nucleic acids and cause damage to other cellular functions such as membrane fluidity, ion transport, enzyme activities, and protein cross-linking (Zelko, Mariana & Folz, 2002).

In normal cells, ROS form as natural byproducts of oxygen metabolism, and they have important roles in cell signaling and homeostasis. Superoxide is the common ROS produced by mitochondria, and .15-2% of cellular oxygen consumption is estimated to result in superoxide production (Hamanaka, Navdeep & Chandel, 2010). Normally after ROS are produced, they are converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD), an enzyme that is part of the cell’s antioxidant defense system. There are three major families of SOD, characterized by their metal content. SOD-1 contains copper/zinc, and is found in the cell’s cytoplasm, nucleus, and within the intermembrane space of the mitochondria. Interestingly, the gene encoding SOD-1 is found on chromosome 21, the same chromosome that is mutated in some cases of AD (Zelko, Mariana & Folz, 2002). SOD-2 is found in the mitochondrial matrix and contains iron/manganese, and SOD-3, like SOD-1 contains copper/zinc though it is found only in the extracellular space.

During times of cellular stress such as in AD, ROS levels can increase dramatically and result in oxidative damage to cells. In general, oxidative stress refers to
the overpowering of the anti-oxidative defense system by the oxidative system. Oxidative stress is recognized as one of the earliest changes in both familial and sporadic AD, and of the different SOD categories, SOD-1 has been shown more frequently than others to affect, and be affected by AD pathogenesis. Murakami et al (2012) found that deletions of SOD-1 significantly accelerates Aβ dependent learning and memory deficits in an age dependent manner by crossing an AD mouse model (Tg2576) expressing human APP (hAPP) with a SOD-1 double knockout mouse model to generate an hAPP SOD-1 deficient mouse. They also found that plaque load was significantly increased in the hAPP SOD-1 deficient mice compared to hAPP mice with normal SOD-1 expression. Additionally, they showed that reduced SOD-1 levels are correlated with increased neuronal inflammation (Murakami, Murata, Noda, Irie, Shirasawa & Shimizu, 2012).

**Inflammation**

In addition to the accumulation of amyloid beta deposits and tau tangles, chronic inflammation is considered a hallmark feature of AD. Markers of inflammation, such as microglia and proinflammatory cytokines TNF-α and IL-1β, are clearly present in brain regions affected by AD and are elevated surrounding plaques. (Vasto, Candore, Listi, Balistreri, Malavolta & Lio, 2008). Microglia, the immune cells of the nervous system, are responsible for mediating inflammation by releasing both anti-inflammatory cytokines, and pro-inflammatory cytokines in response to insult (both physical and biological). In general, the inflammatory response is meant to protect tissue against further damage, however, if the inflammation lasts for long periods of time, it can actually become harmful (Lull & Block, 2010).
It is currently unclear whether or not inflammation plays a role in the development of AD by triggering Aβ accumulation, or if it is the result of AD. Various studies have found that patients who have a history of serious head injury, resulting in inflammation, are at increased risk for developing AD, and patients who suffer from inflammatory diseases such as rheumatoid arthritis are also at increased risk for AD (Sivanandam & Thakur, 2012). Additionally, people that take anti-inflammatory drugs, such as NSAIDs, on a regular basis are less likely to develop AD (Cote et al., 2012). Studies such as these suggest that inflammation itself can be a risk factor for AD.

Other research has shown that protein misfolding, such as in the amyloidogenic pathway, can activate inflammatory pathways, which once activated, continue to remain activated through positive feedback loops. It isn’t clear why, but somehow this chronically activated inflammatory signaling then has the ability to elevate the presence and activity of β-secretase, thereby increasing the rate of Aβ production via the amyloidogenic pathway (Heneka et al., 2005). One such pro-inflammatory cytokine is TNF-α which is secreted by microglia surrounding plaques, and plays a key role in initiating and regulating the production of other cytokines during inflammation.

Because chronic inflammation can further exacerbate AD by increasing the rate of Aβ production, and activating apoptotic pathways in both damaged and healthy tissue, Shi et al. attempted to reduce levels of inflammation in an APP/PS1 mouse model by injecting a TNF-α antibody. Their results revealed that treatment with TNF-α antibody,
Infliximab, not only reduced levels of TNF-α expression, but also reduced the amount of amyloid plaques in the brain (Shi et al., 2011).

**Current Study**

The overall goal of this study was to investigate the role of dietary copper and zinc homeostasis on inflammation and oxidative stress in an early onset mouse model of AD. While many studies have linked dietary metal dyshomeostasis to AD, as well as oxidative stress and inflammation to AD, this is the first study to look at markers of inflammation and oxidative stress and metal dyshomeostasis in AD. The ability to do this was accomplished by creating 6 different experimental groups: 3 diet conditions and 2 genotypes. Animals were fed either a copper control diet (16 ppm copper) or a copper deficient diet (4 ppm copper), and were given either lab drinking water or zinc enhanced drinking water (10 ppm zinc). For a list of diet combinations administered see Table 1.

To generate an early onset model of AD, transgenic “J20” mice were bred with wildtype C57 mice. The J20 mouse model of AD overexpresses 2 hAPP mutations (Swedish + Indiana) driven by the PDGFB promoter, which cause the mice to begin developing Aβ plaques as early as 6 months old. Previous work in our lab used CRND8 mice as an early onset model of AD. The present study switched to the J20 model for a number of reasons including availability, background strain, and visual ability. Like J20 mice, CRND8 mice also express Swedish and Indiana mutations of hAPP and develop Aβ plaques by 6 months, however, they are generated on a different background strain than the wildtype animals used by our lab, and they tend to have visual deficits which interfere with tests of spatial memory. Our lab has also utilized the Tg2576 mouse model
of AD in the past, but this mouse model only expresses the Swedish mutation of hAPP, and doesn’t develop plaques until 11-13 months.

The specific hypotheses tested in this experiment are as follows:

**Hypothesis 1:** J20 animals will have higher overall levels of SOD-1 expression compared to WT mice.

**Hypothesis 2:** Animals raised on a copper deficient diet will have similar levels of SOD-1 expression to animals raised on a zinc enhanced diet. Both will have higher levels of SOD-1 expression than animals raised on copper control diet.

**Hypothesis 3:** The combination of genotype and diet condition will reveal significant differences in SOD-1 expression. J20 copper deficient and J20 zinc enhanced mice will have similar levels of expression, and both will have the highest levels of SOD-1 compared to all other groups. Following will be WT copper deficient and WT zinc enhanced. The lowest levels of SOD-1 expression will be J20 copper control followed by WT copper control, with J20 copper control having higher levels of expression than WT copper control.

**Hypothesis 4:** J20 animals will have higher overall levels of TNF-α expression compared to WT mice.
**Hypothesis 5:** Animals raised on a copper deficient diet will have similar levels of TNF-α expression to animals raised on a zinc enhanced diet. Both will have higher levels of TNF-α expression than animals raised on copper control diet.

**Hypothesis 6:** The combination of genotype and diet condition will reveal significant differences in TNF-α expression. J20 copper deficient and J20 zinc enhanced mice will have similar levels of expression, and both will have the highest levels of TNF-α compared to all other groups. Following will be WT copper deficient and WT zinc enhanced. The lowest levels of TNF-α expression will be J20 copper control followed by WT copper control, with J20 copper control having higher levels of expression than WT copper control.

**Table 1. Experimental Groups**

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<tr>
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<th>J20</th>
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<tr>
<td><strong>Zinc Enhanced</strong></td>
<td>Zinc Water+ Copper Control Diet</td>
<td>Zinc Water+ Copper Control Diet</td>
</tr>
<tr>
<td><strong>Copper Deficient</strong></td>
<td>Lab Water+ Copper Control Diet</td>
<td>Lab Water+ Copper Control Diet</td>
</tr>
<tr>
<td><strong>Copper Control</strong></td>
<td>Lab Water+ Copper Deficient Diet</td>
<td>Lab Water+ Copper Deficient Diet</td>
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CHAPTER TWO: MATERIALS AND METHODS

Animals

All experiments using mice were performed with the approval of the George Mason University Institutional Animal Care and Use Committee. Female breeders included n=15 wildtype C57BL/6J (“B6”) mice purchased from the Jackson Laboratory, housed three to a cage. Male breeders included n=5 transgenic J20 mice also purchased from the Jackson Laboratory, housed individually. Male breeders carry a hemizygous mutation for both the Swedish and Indiana mutations of hAPP and were crossed with wildtype females to produce offspring in which 50% would also carry the hAPP gene thus comprising an “early onset” AD group. The other 50% of offspring not carrying the hAPP gene comprised the wildtype control group.

Because of limited space, breeding occurred in three rounds, yielding three experimental cohorts. During each round of breeding, three female mice were paired with one male mouse in his home cage, for two weeks. After this two-week period female breeders were housed individually for the length of their gestational period (21 days). At postnatal day 10-21 pups were genotyped by tail snip analysis. At this age the tail snipping procedure is not painful, and does not require anesthesia. Approximately 5mm was snipped from the tail of each mouse by the use of surgical scissors sterilized in 70% ethanol. Samples were collected from each animal and placed in a sterile 96 count well
plate that was then sent to Transnetyx Laboratories for genotype analysis by PCR. Upon receiving the results of the genotype analysis, at postnatal day 21 pups were weaned from their mothers and group housed (2-4/cage) according to sex and genotype.

The animal colony was maintained on a 12:12 light/dark schedule and temperature was maintained between 68-75 degrees with humidity between 45-65%. All cages used Tek-FRESH bedding and were cleaned once a week by SoBran Inc. Each cage contained a running wheel for exercise, and an igloo for housing/nesting. In an effort to avoid increased attrition rates typical of this mouse model, all involved personnel were thoroughly trained in sterile lab techniques.

**Food/Water**

All breeder males and females were fed a standard lab animal diet (7012) ad libitum, purchased from Harlan Industries. Two weeks prior to pairing breeders, both males and females were given Love Mash Rodent Reproductive Diet (BioServ), to increase the likelihood of reproduction. Experimental animals were fed a specialized diet formulated by Harlan to control for copper intake. The two specialized diets used were copper control (16 ppm copper) and copper deficient (4 ppm copper). Other than the varying levels of copper, the two diets used were identical. All experimental animals were given copper control diet until 2 months of age, at which point approximately 1/3 of them were switched to copper deficient. It is also at this point that another 1/3 of them were switched from lab drinking water to zinc enhanced drinking water (for the total number of animals on each diet, see Table 2).
Animals in the zinc-enhanced group were given drinking water containing 10 ppm zinc carbonate (ZnCO\textsubscript{3}). The zinc enhanced water was be prepared by dissolving 10,000 ppm zinc in 5% nitric acid (HNO\textsubscript{3}), and buffered to a PH level of 7 using sodium bicarbonate (Na\textsubscript{2}CO\textsubscript{3}). All waters were made and stored in separate polycarbonate carboys. Water samples were taken regularly and analyzed for metal content at the United States Geological Survey (USGS) in Reston, Virginia. Food and water consumption was recorded by weight bi-weekly and logged in a colony maintenance binder.

Table 2. Total Number of Animals per Experimental Group

<table>
<thead>
<tr>
<th></th>
<th>Cu Control + Lab Water</th>
<th>Cu Deficient + Lab Water</th>
<th>Cu Control + Zn Water</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Wt</td>
<td>n=15</td>
<td>n=17</td>
<td>n=16</td>
<td>n=48</td>
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<tr>
<td>Tg</td>
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<td>n=13</td>
<td>n=40</td>
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<tr>
<td>Total</td>
<td>n=29</td>
<td>n=30</td>
<td>n=29</td>
<td>n=88</td>
</tr>
</tbody>
</table>

Western Blotting

At 6.5-7 months of age, experimental animals were sacrificed by gradual CO\textsubscript{2} asphyxiation and subsequent decapitation. The brains were then extracted and flash-frozen on dry ice. Before decapitation tail pinches and whisker deflection were performed on each animal to ensure that the animals were deeply anesthetized. Within an hour of extraction, brains were moved to a -80° Celsius freezer and stored until all three cohorts.
were completed. Only a subset of total animals bred were used for the biochemical analyses in this thesis, but all animals bred were used in another study to test spatial memory ability. Results of these behavioral analyses are reviewed briefly in the discussion section.

At the end of the third cohort, 5 animals (mixed sex) were selected at random from each of the six experimental conditions, and the brains were hemisected into left and right hemispheres. Additionally the cerebellum was removed and discarded, and the prefrontal cortex was removed from the right hemisphere. The left hemisphere was placed back into the -80°C Celsius freezer for subsequent analysis by Congo Red staining, and the right hemisphere was homogenized for Western blot analysis.

The right hemisphere from each selected animal was homogenized in RIPA lysis buffer using a glass dounce tissue grinder. Protease inhibitors (Roche) and phosphatase inhibitors (Pierce) were added to the RIPA lysis buffer to prevent proteolysis and preserve the phosphorylation state of relevant proteins. Homogenates were transferred to 1.5 ml eppendorf tubes and centrifuged at 14,000 RPM for 15 minutes; the supernatant was then collected and separated into 100 ul aliquots. Protein concentration of the supernatant was calculated using the Pierce BCA protein assay, and the concentration of protein used for Western blot analysis was 100 ug of protein per animal/lane. Immediately upon completion of the BCA assay, samples were prepared for polyacrylamide gel electrophoresis (PAGE-SDS) by the addition of 4X NuPAGE LDS Sample Buffer (Invitrogen) and 10X NuPAGE Sample Reducing Agent (Invitrogen). Next, samples were heated in an 80°C Celsius water bath for 5 minutes, and then loaded
onto NuPAGE Novex 4—12% Bis-Tris-gels (Invitrogen). Samples were run at 120 volts for approximately 90 minutes.

Gels were transferred to nitrocellulose membranes using the iBlot 2 Dry Blotting System (Invitrogen) set to program 3, which passes 20 volts of current through the gel/membrane stack for a total of 7 minutes. Membranes were then blocked for 45 minutes in a 3% blotto solution containing tris-buffered saline and Tween-20 (TBST) with 3% milk powder, and incubated with primary antibody overnight on an agitator at 4°C. The next morning membranes were washed in TBST 3 times for 20 minutes and incubated with HRP-conjugated secondary antibody at room temperature for 90 minutes. Membranes were then washed again in TBST, 3 times for 20 minutes, and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and imaged using G:Box Chemi-XT4 GENEsys software (Syngene). Membranes were incubated with the chemiluminescent substrate for 3 minutes then exposed and imaged every 30 seconds for a total of 10 minutes.

After imaging, band density was determined using ImageJ (NIH) and analyzed by two-way ANOVA. The dilutions for the primary antibodies were 1:500 for TNF-α (Bioss) and 1:1000 for SOD-1 (Thermo Scientific). The dilution for the HRP-conjugated secondary antibody was 1:10,000 (Jackson ImmunoResearch). Additionally, β-actin was used at a dilution of 1:1,000 (Cell Signal) as a loading control.

**Congo Red Staining**

To measure the degree of plaque formation, Congo Red staining was performed in a subset of the transgenic mice. A total of 2 brains were taken from each diet condition in
the J20 animals only (n=6), sliced at 25 microns, and mounted onto charged glass slides. The Jackson Laboratory states that the highest level of plaque formation will occur in the neocortex and hippocampus, thus slices were collected from 3 different ROI’s: ventral hippocampus (vHC), dorsal hippocampus (dHC), and anterior hippocampus (aHC).

Figure 2. ROI 1= vHC (Bregma -3.16 mm)

Figure 3. ROI 2= dHC (Bregma -1.94 mm)
After slicing, sections were dried overnight at room temperature. The next day, slides were placed in Mayer’s Hematoxylin for 10 minutes and then washed in tap water until the tissue appeared blue. Slides were then placed in a working sodium chloride solution for 20 minutes. Next, slides were moved to a working Congo Red solution for 1 hour, dehydrated in alcohol, cleared in xylene, and cover-slipped. Cover-slipped slides were allowed to dry overnight before being imaged under an Olympus BX51 fluorescent/polarizing light microscope.
CHAPTER THREE: RESULTS

SOD1 Expression

Results of a 2x3 ANOVA performed for SOD1 revealed no main effect of genotype on SOD1 expression ($F(1, 24)=.472$, $p=.499$), no main effect of diet condition ($F(2, 24)=.910$, $p=.416$), and no interaction ($F(2, 24)=.186$, $p=.832$). This indicates that levels of oxidative stress, as measured by SOD1 expression, are relatively equal between WT and J20 animals, and relatively equal between the 3 diet conditions (See Table 2).

Although the data weren’t significant, the zinc-enhanced group had the highest mean SOD1 expression for both J20 and WT animals, followed by animals raised on copper deficient diet. The copper control group had the lowest mean SOD1 expression in both J20 and WT animals (Figure 5). Regardless of diet condition, J20 animals had higher overall levels of SOD1 compared to WT animals ($F$).

Additionally, the data were analyzed using a 2x2x2 ANOVA for SOD1, where the diet was split into food type (copper control food and copper deficient food) and water type (zinc enhanced water and lab water). Results revealed no main effect of food type ($F(1, 24)= 1.03$, $p=.320$), water type ($F(1, 24)= 1.63$, $p=.214$), or genotype ($F(1, 24)= .644$, $p=.430$), indicating that levels of oxidative stress are the same between animals raised on copper control food (16ppm) and copper deficient food (4ppm). This also indicates that animals raised on 10ppm zinc water do not differ in their levels of oxidative
stress compared to animals raised on lab water. Again, levels of oxidative stress are relatively equal between J20 and WT animals.

Table 3. 2x3 ANOVA Mean Densities, SOD1

<table>
<thead>
<tr>
<th></th>
<th>J20</th>
<th>WT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD (±/-)</td>
<td>N</td>
</tr>
<tr>
<td>Zinc Enhanced</td>
<td>1.12</td>
<td>.35</td>
<td>5</td>
</tr>
<tr>
<td>Copper Deficient</td>
<td>1.11</td>
<td>.21</td>
<td>5</td>
</tr>
<tr>
<td>Copper Control</td>
<td>0.94</td>
<td>.12</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>1.05</td>
<td>.24</td>
<td>15</td>
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</tbody>
</table>

Figure 5. SOD-1 Expression by Diet
Zinc enhanced animals had the highest mean SOD-1 expression and copper control animals had the lowest mean SOD-1 expression. Copper control animals were intermediate. Values reflect densitometry and are in arbitrary units. Bars represent SOD-1 averages ± SD.
F. SOD-1 Expression by Genotype
Animals with a double hAPP mutation have higher levels of mean SOD-1 expression. Values reflect densitometry and are in arbitrary units. Bars represent SOD-1 averages ± SD.

TNF-α Expression

A 2x3 ANOVA was performed to examine TNF-α expression, which revealed no significant difference across genotype ($F(1, 24)= 1.6, p=.218$) or diet condition ($F(2, 24)= .3, p=.744$), indicating that levels of inflammation, as measured by TNF-α expression, are the same between WT and J20 animals, and the same between animals raised on the 3 different diet types. There was also no interaction between genotype and diet condition ($F(2, 24)= .141, p=.870$) (See Table 3). Although there was no main effect of genotype, J20 animals had higher levels of TNF-α expression compared to WT, represented by mean band density (Figure 7).

In WT animals, those raised on a copper deficient diet had the highest level of TNF-α expression, followed by copper control. Zinc-enhanced animals had the lowest
level of TNF-α expression. In J20 animals, those raised on a zinc enhance diet had TNF-α levels identical to those raised on a copper deficient diet, and copper control animals had the lowest level of TNF-α expression (Figure 8).

When analyzed using a 2x2x2 ANOVA, there was also no significant effect of food type \((F(1, 24)=.60, p=.447)\), water type \((F(1, 24)=.127, p=.724)\), or genotype \((F(1, 24)= 1.70, p=.204)\). This suggests that levels of inflammation are not different between copper control diet (16 ppm) and copper deficient diet (4 ppm) and that inflammation is not different between animals raised on zinc enhanced drinking water (10 ppm) and lab water. Levels of inflammation are the same between WT and J20 animals.

**Table 4. 2x3 ANOVA Mean Densities, TNF-α**

<table>
<thead>
<tr>
<th></th>
<th>J20</th>
<th>WT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD (+/-)</td>
<td>N</td>
</tr>
<tr>
<td>Zinc Enhanced</td>
<td>.91</td>
<td>.15</td>
<td>5</td>
</tr>
<tr>
<td>Copper Deficient</td>
<td>.91</td>
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<td>5</td>
</tr>
<tr>
<td>Copper Control</td>
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<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>.88</td>
<td>.19</td>
<td>15</td>
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</tbody>
</table>
Figure 7. TNF-α Expression by Genotype
Animals with a double hAPP mutation have higher levels of mean TNF-α expression. Values reflect densitometry and are in arbitrary units. Bars represent TNF-α averages ± SD.

Figure 8. TNF-α Expression by Diet
Copper deficient and zinc enhanced animals had the highest levels of mean TNF-α expression; copper control animals had the lowest levels of mean TNF-α expression. Values reflect densitometry and are in arbitrary units. Bars represent TNF-α averages ± SD.
Congo Red Staining

After examining Congo Red stained slides under an Olympus BX51 fluorescent/polarizing light microscope, little plaque formation was observed all any of the transgenic animals across all ROIs (Figures 2-4; plaques circled in yellow).

Figure 9. Congo Red Copper Control Animals (left to right- dHC, vHC, aHC)
Figure 10. Congo Red Copper Deficient Animals (left to right- dHC, vHC, aHC)

Figure 11. Congo Red Zinc Enhanced Animals (left to right- dHC, vHC, aHC)
CHAPTER FOUR: DISCUSSION

In general, amyloid plaques can be categorized as diffuse or dense-core. In the preclinical stages of AD, most plaques found in the brain are the diffuse type, and as the disease progresses the number of diffuse plaques tends to decrease as the number of dense-core plaques increases (Dickson & Vickers, 2001). Diffuse plaques are also often seen in cognitively unimpaired individuals as a normal part of aging. In both plaque types, the main deposit is Aβ, however, dense-core plaques are surrounded by diseased neurons with dystrophic neurites, and diffuse plaques are not (Hutton & Hardy, 1997). Additionally, dense-core plaques tend to have a large number of surrounding microglia, suggesting the activation inflammatory signaling pathways. While microglia are sometimes seen in regions surrounding diffuse plaques, microglial reactions tend to be fewer and weaker (Dickson, 1997).

As observed by Congo Red staining, the lack of dense-core plaque formation in the J20 animals indicates that 6 months may be too early to see any significant neuropathological expression of the hAPP transgene. The Jackson Laboratory states in their strain information for the J20 mouse model that, “diffuse plaque formation is apparent in animals around 5-7 months, and that plaque deposition is progressive, with all J20 mice exhibiting plaques by 10 months” (The Jackson Laboratory, 2014). It’s possible
that J20 mice at 6 months are still in a “preclinical stage” of AD, and therefore it is too early to detect any inflammatory or oxidative changes in these animals.

Several studies have shown that the level of amyloid beta accumulation positively correlates with levels of oxidative stress; as the amount of Aβ increases, so do the levels of ROS such as hydrogen peroxide and nitric oxide. Thus, if J20 animals at 6 months are not yet expressing high levels of Aβ, it is likely that they also are not yet experiencing a detectable increase in markers for oxidative stress.

Morris Water Maze (MWM) data in these animals was collected as part of another thesis study, and found significant behavioral differences between J20 and WT animals. Specifically, WT animals had significantly faster escape latencies across days, suggesting their ability to learn the spatial navigation task more quickly than J20 animals. On probe trial days, WT mice spent significantly longer in the goal quadrant, and had significantly more platform crossings than J20 animals. This suggests that WT mice were better able to remember the location of the platform based on spatial cues than the J20 animals.

Considering the significant behavioral data and lack of significant biochemical data, it’s possible that behavioral deficits manifest in AD more quickly than neuropathological deficits. This is supported by a study conducted by Billings et al. in 2005 which found impairments in long-term memory retention in a 3xTg AD mouse model at 4 months of age, but no Aβ plaque or NFT pathology. Billings et al. propose that intraneuronal Aβ accumulation is to blame for the memory deficits seen at this age, rather than the extracellular Aβ plaques observed later in AD pathology (Billings et al.,
2005). It’s possible that intraneuronal Aβ contributes to the behavioral deficits early in AD and that extracellular Aβ plaques exacerbate the behavioral impairment, and contribute to inflammation and oxidative stress later in AD.

While significant genotype differences observed in MWM, no significant main effect of diet was observed, which is consistent with the biochemical data in this thesis. It’s possible that no diet effect was seen in these animals because the copper deficient diet formulated for our lab is only mildly deficient. At 4ppm copper, levels may not be low enough to induce oxidative stress or inflammation. Other studies looking at copper deficiency in rodents use copper deficient diets that fall as low as .5-ppm copper (Prohaska & Smith, 1982). Furthermore, at 10ppm zinc, the zinc-enhanced drinking water may not have been concentrated enough. In fact, some studies indicate that low levels of zinc supplementation can actually be protective by reducing levels of inflammation and oxidative stress (Prasad et al., 2007), and it has even been suggested as an immediate treatment option for those who suffer traumatic brain injury (Cope et al., 2012).

Initially this thesis aimed to include immunohistochemistry to spatially map the proteins of interest, however, due to a massive amount of method development that went into optimizing Western blotting, tissue samples will be analyzed by immunohistochemistry at a later date. Many issues arose throughout the course of Western blotting that hadn’t been anticipated, and likely would have been avoided had our lab not lost Dr. Dan Cox, a Krasnow Institute researcher who accepted a job at Georgia Tech University around the start of this project.
Dr. Flinn’s lab had been trained to perform Western blotting to look at simple housekeeping proteins, such as β–tubulin and β–actin, which are expressed at high levels making it very easy to probe for these proteins. It was unknown at the start of this project, that not all proteins of interest can be probed for using the same Western blot protocol. One of the proteins we would have liked to probe for is Nuclear Factor kappa B (NFkB), a protein that is part of a major inflammatory pathway that is activated by TNF-α. Unlike TNF-α and SOD1, which are cytosolic proteins, NFkB is a nuclear protein, therefore in order to probe for it, the tissue homogenate needs to be treated differently from the beginning of the project and the proteins extracted by differential centrifugation. At the time of tissue processing, the tissue homogenate for this thesis was not processed in such a way that nuclear proteins could not be probed for, and this wasn’t learned until multiple attempts to probe for NFkB failed.

Another issue we ran into during this project was our phosphatase inhibitors interfering with our BCA protein assay. It was impossible to generate a standard curve to measure our brain tissue samples against, because all of the known protein dilutions (RIPA lysis buffer was used as the diluent), which were made to generate a standard curve, were measuring at the same level of absorbency by spectrophotometry, indicating that they all had the same amount of protein in them. It took several weeks and multiple emails with Dr. Cox to discover that the phosphatase inhibitor added to the RIPA lysis buffer was the cause of the problem. From this point on dH2O was used as the diluent for the BCA assay, and we were able to generate a standard curve. These issues were just a few among many other that interfered with timeline of this project. With the help of Dr.
Cox and Dr. Nadine Kabbani’s lab, we were able to successfully probe for TNF-α and SOD1, and optimize a Western blot protocol for future work in our lab.

Future research in our lab will aim to analyze behavioral deficits in J20 mice that have been crossed with E4 mice at 6, 9, and 12 months of age, as well as analyzing brain tissue at 12 months for markers of inflammation and oxidative stress. With the addition of the E4 gene, our lab will be able to examine these markers in a late onset model of AD, which is especially useful as the majority of AD cases occur after age 65. Not only will biomarkers be analyzed in this mouse model, activities of daily living and circadian rhythm activity will also be assessed. In addition to utilizing Western blotting as a marker of protein expression, future work will aim to look at proteins by immunohistochemistry and Western blot, so that proteins can be quantified and spatially mapped.
REFERENCES


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BIOGRAPHY

Katelyn Boggs received her Bachelor of Science from George Mason University in Spring 2012. As an undergraduate she majored in Psychology and minored in Neuroscience. Upon receiving her B.S. degree, she began graduate school at George Mason University in Fall 2012 and began researching Alzheimer’s disease under the guidance of Dr. Jane Flinn. She intends to continue her graduate career in Psychology and pursue a doctorate degree from George Mason University.