AN INVESTIGATION INTO THE ROLE OF COPPER DEFICIENCY AND ZINC IN A MOUSE MODEL OF EARLY ONSET ALZHEIMER'S DISEASE

by

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of
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An Investigation into the Role of Copper Deficiency and Zinc in a Mouse Model of Early Onset Alzheimer's Disease.

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Bachelor of Science
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DEDICATION

I would like to dedicate this thesis to my family. First, to my parents, without their love, support and encouragement throughout the years, I never would have made it to where I am today. Second, to my husband, who has supported me unconditionally through receiving my B.S. in another country and starting graduate school with a newborn baby girl. Third, to my daughter, who unknowingly gives me the strength and courage every day to be the very best mom, wife, student, and role model that I can be.

Lastly, and most importantly, I would like to dedicate the product of this research to those that strive to age gracefully despite the hurdles that aging itself creates. Specifically, to my grandmother Jean Marie Haack; without her, I would not have gone into the field of work that I am in. May we soon find a cure.

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LIST OF ABBREVIATIONS AND SYMBOLS

Alzheimer's Disease……AD
Amyloid Beta……...….Aβ
(Human) Amyloid Precursor Protein……(h)APP
Copper……...……Cu
Iron.....FE
Morris Water Maze……MWM
Novel Object Recognition…….……NOR
Transgenic……...…..Tg
Wildtype……...….Wt
Zinc……Zn

ABSTRACT

AN INVESTIGATION INTO THE ROLE OF COPPER DEFICIENCY AND ZINC IN A MOUSE MODEL OF EARLY ONSET ALZHEIMER'S DISEASE

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George Mason University, 2014

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While significant advances have been made in understanding the neural mechanisms of Alzheimer's disease (AD), many aspects remain unknown. More recent theories revolve around the effects of metals in the diseased brain. Building on research previously conducted in our lab, this study examined the roles of two metals, zinc (Zn) and copper (Cu), in a mouse model of early onset AD. The previously mentioned studies found that excess Zn caused behavioral impairments in rats and mice; however, the addition of Cu to the Zn-enhanced water remediated the negative effects seen in the purely Zn group. This supports the theory that the effects of "excess Zn" were due to an induced Cu deficiency. To test this theory it was necessary to look at a Cu deficiency directly. For this purpose, we, together with Harlan laboratory nutritionists, developed a specialized Cu deficient and Cu control diet (differing only in levels of Cu). Wildtype (Wt) (C57Bl/6J) and transgenic (Tg) mice with one copy of a doubly mutated human

amyloid precursor protein (hAPP) gene (J20; breeders obtained from the Jackson Laboratory) were raised in one of three groups: a strictly control group (lab water + control diet), or one of two experimental conditions involving excess Zn (Znwater + control diet) and a diet deficient in Cu (Cu deficient diet + lab water). Mice were run in two behavioral tasks, novel object recognition (NOR) and Morris water maze (MWM), aimed at identifying the effects of the metals on memory deficits seen early in AD patients. Mice were tested beginning at 6 months of age. All Wt and Tg groups distinguished the novel object at 15-minutes, groups varied widely at 1 hour, and no differences were seen at 24-hours. In MWM, there was a significant difference between Wt and Tg mice on all measures. Cu-deficient Tg mice were trending toward a significant difference in latency on day 3 from the Zn-enhanced group, who were slower to learn the task. Within the Tg group (MWM), Cu deficient mice unexpectedly performed the best overall, the controls performed intermediately and the Zn-enhanced group showed the worst performance. The differing patterns seen in MWM and NOR indicate that the effects of Cu and Zn are dependent upon different brain structures, and that the Zn effect is not entirely due to an induced Cu deficiency.

CHAPTER ONE: INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, accounting for approximately two-thirds of all documented cases (Jonsson et al., 2013). By 2030, the United States' (U.S.) population of aged individuals (65 years and older) is expected to double, reaching nearly 70 million people (Plassman et al., 2007). With such exponential growth expected, more emphasis is being put on chronic diseases associated with aging, including AD (2007). Two forms of AD exist: late onset, which occurs in those over 60 years of age, and early-onset, or familial AD, occurring in mid-life. While both forms have a genetic component, the late-onset gene is merely a "risk-factor"; it is likely that the late-onset form also has an environmental, and lifestyle component (Moceri et al., 2001). Conversely, the rare, early-onset form has specific inheritable mutations on three genes, the inheritance of any one of which will almost certainly lead to AD (Levy-Lahad & Bird, 1996; Di Fede et al., 2009). Understanding AD is at the forefront of an effort to increase research on diseases of aging due to the severe cognitive decline and loss of independence associated with its progression; it is a disease with no current cure and millions of potential victims.

The primary neuropathological features of AD are intracellular neurofibrillary tangles, consisting of hyperphosphorylated tau protein, and extracellular neuritic plaques, comprised of amyloid beta (Aβ) protein, after its sequential cleavage from the larger

amyloid precursor protein (APP). APP can be cleaved in two alternate ways, referred to as the nonamyloidogenic and amyloidogenic pathways. In the nonamyloidogenic pathway, the transmembrane protein APP is cleaved sequentially by alpha and gamma secretases, releasing the P3 peptide. Conversely, sequential cleavage of APP by beta and gamma secretases causes the formation of the Aβ peptide (Hung, Bush & Cherny, 2010). It is a widely accepted theory that the Aβ peptides found in AD plaques play a causal role in development of the disease and the cognitive decline that follows ("amyloid cascade" theory) (Roberson et al., 2007; Harris et al., 2010; Jonsson et al., 2013). The plaques and tangles that form in the brains of AD patients generally cause severe, progressive cognitive and personality deficits, as well as neuronal atrophy, resultant from synaptic loss in such brain areas as the entorhinal cortex, hippocampus, neocortex, and amygdala (Hung et al., 2010)

Further, it has been documented that the Aβ protein has a high binding affinity for metals, including Cu, Zn, and Fe, which are also found in high concentrations within AD plaques (Bush, 2003). The concentrations of these metals in grey matter are substantial, indicating that they play crucial roles in biological processes (Bush & Tanzi, 2002). Non-physiological levels result in a breakdown of the mechanisms that they regulate; this includes production of reactive oxygen species and subsequent oxidative stress (Shcherbatykh & Carpenter, 2007). Metals in brain have also been linked to regulating neurotransmission, synaptogenesis, neurogenesis, neurite outgrowth, neurotransmitter biosynthesis, oxidative phosphorylation and oxygen transport (Hung et al, 2010). Thus, increasing emphasis has been put on the roles of these metals in AD, with results

suggesting that the dyshomeostasis of these metals has a significant effect on Aβ accumulation. For the general population, who now has access to vast amounts of information regarding dietary means to increase "healthy aging" and "healthy life expectancy", this can lead to consumption of multivitamins and enriched foods. Currently, over half (52%) of older individuals regularly partake in dietary supplementation, with increases after age 60 (63%) (Radimer et al., 2004). Little information is provided that indicates a potentially harmful effect of too much supplementation. For those who have neurodegenerative disorders, too much supplementation may result in a more rapid expression and/or progression of those disorders (specific to this thesis, certain types of metals). Along with intentional exposure, there is a potential risk of incidental exposure through medications, ointments or even drinking water. Cu pipes used for plumbing cause measureable increases in Cu in drinking water (Dietrich et al., 2004). Further, a common treatment for age-related macular degeneration contains excess levels of Zn, as does denture cream (AREDS, 2002; Hedera et al., 2009). For the scope of this thesis, the roles of Zn and Cu in AD neuropathology were evaluated.

Under normal conditions, unbound, or "free", Zn is concentrated in synaptic vesicles. When the synapse is activated, Zn is released into the cleft where it is able to react with various postsynaptic receptors and/or channels; the Zn is then returned to the vesicles via Zn transporters, specifically ZnT3, which has been found to decrease in the AD brain (Lee, Cho, Seo, Hwang, & Koh, 2012; Bjorklund et al., 2012). Synaptic Zn is involved in many crucial biological processes including the activation of presynaptic Erk-

dependent signaling, crucial for proper hippocampal memory function and regulating synaptic plasticity and long- term potentiation, as well as modulating calcium channels, AMPA and NMDA receptors (Sindreu, Palmiter, & Storm, 2011; Li, Hough, Frederickson, & Sarvey 2001; Adlard, Parncutt, Finkelstein, & Bush, 2010; Bjorklund et al., 2012). Concerning the formation of AD plaques, Zn stabilizes toxic Aβ oligomers and targets them to the postsynaptic region (Bjorklund et al., 2012). Additionally, Zn can interact with the N-terminal of Aβ, inducing conformational changes that may facilitate the formation of oligomers (2012). Due to its involvement in such a number of processes, changes in synaptic Zn levels can seriously alter synaptic transmission (2012).

Synaptic Zn, specifically, also shows a correlation with AD neuropathology. Genetic ablation of the Zn transporter ZnT3 caused a lack of vesicular Zn and a decrease in Aβ deposits in mice with mutated hAPP (Friedlich et al., 2004; Lee et al., 2002). Aggregation of Aβ is highest at excitatory synapses, where Zn release is increased during neurotransmission (Deshpande, Kawai, Metherate, Glabe, & Busciglio, 2009; Stoltenberg et al., 2007). Other research has found that, due to the loss of ZnT3 with age, an age-dependent loss of Zn uptake may lead to cognitive loss through extracellular Aβ being aggregated by and capturing synaptic Zn, as shown in ZnT3 knockout mice that lack a synaptic zinc pool (Adlard et al., 2010). With a focus on metallostasis in AD, it has been suggested that metal chelators may be a promising therapeutic agent, by removing metals from the synapse. Long-term administration of certain metal chelators has significantly reduced the levels of synaptic Zn as well as amyloid deposits in hAPP mice as compared to mice given vehicle treatment (Lee, Friedman, Angel, Kozak, & Koh, 2004). Lee et al.

specifically used the metal chelator DP-109 daily for 3 months in female hAPP mice (2004). Female Tg mice given DP-109 had drastically reduced plaque burden when compared to the mice receiving the vehicle (2004).

A majority of work done with transition metals has focused on biochemical assays, so there is much less literature from a behavioral standpoint; however, Zn has been linked to altered fear response and spatial memory in rats, and spatial memory deficits in mice with mutated hAPP (Flinn et al., 2005; Chrosniak, Smith, Flinn, McDonald, & Jones, 2006; Railey, Micheli, Wanschura, & Flinn, 2010; Linkous, Adlard, Wanschura, Conko, & Flinn, 2009; Railey, Groeber, & Flinn, 2011). In 2005, Flinn et al. showed both reference and working memory impairments (Morris water maze; MWM) in rats treated pre- and postnatally with drinking water enhanced with 10ppm of Zn carbonate. Linkous et al. investigated the role of Zn in AD by using two strains of Tg mice (with differing hAPP mutations) compared to Wt littermates (2009). Spatial memory deficits, as seen in the MWM, were greatest in the Zn-enhanced Tg groups, although Wt animals on Zn water also showed impairments. Deficits caused by Zn administration in the Tg animals were greater than the normal transgene effect (2009).

Three of the previously mentioned behavioral studies found that the addition of Cu to the Zn-enhanced water remediated the negative effects seen in the Zn-enhanced group (Chrosniak et al. 2006; Railey et al., 2010; Railey et al., 2011). Chrosniak et al. showed that Zn administration in rats, both pre- and postnatally, potentiated an inability to retain fearful memories (2006). Rats were also raised on water enhanced with Zn+Cu and tested for spatial memory (MWM). There were significant improvements in the

Zn+Cu group, as compared to the Zn group, indicating that the Cu may have remediated some of the negative behavioral effects seen in the purely Zn group (2006). In a similar experiment done by Railey et al. (2010), rats raised on Zn water showed significantly higher freezing rates during contextual retention and extinction as well as cued extinction. In MWM, the Zn supplemented group showed significantly longer latencies. The addition of Cu to the Zn water brought that group's freezing and latencies much closer to those of controls (2010). Lastly, in a study with Tg mice (hAPP mutation), Railey et al. used lab water control, enhanced Zn, enhanced Fe, and enhanced Zn+Cu groups to further investigate the roles of metals in AD (2011). Mice that were raised on Zn and Fe showed significantly longer latencies and fewer platform crossings, while there was no significant difference between the Zn+Cu and control groups. Taken together, this behavioral data supports the notion that Zn may interact with Cu to precipitate neuropathological changes, especially where AD is concerned.

Cu is thus another transition metal implicated in AD. The mammalian brain is very rich in Cu, much more so than any other organ in the body, and has a much higher ion concentration than the blood, which is the main biological fluid for Cu transport (Zatta & Frank, 2007). The human brain, specifically, contains the highest Cu concentration when levels are compared to other species (2007). While the exact mechanisms of Cu are unknown, it is clear that under normal conditions Cu stabilizes superoxide dismutase 1 (SOD1), thereby protecting against the generation of reactive oxygen species (Bayer et al., 2003). It is also implicated in iron transport, which is another metal dysregulated in AD, is essential for a number of enzymes required for CNS

cellular metabolic functions (ceruloplasmin, cytochrome c oxidase) and is important for myelination.

In a study by Bayer et al., overexpression of APP in Tg mice reduced SOD1 activity when compared to wildtype littermates, which allowed for the generation of reactive oxygen species; this was remediated by Cu treatment (2003). Cu treatment also lowered Aβ in the CNS before there was a detectable reduction in plaques (2003). Further, SOD1 deficiency has been shown to aggravate Aβ protein oligomerization and memory loss in AD Tg mice (Murakami et al., 2011). The abnormal characteristics seen in the mice were essentially caused by oxidative damage resultant from the absence of SOD1 functionality (2011). SOD1 deficiency also spurred tau phosphorylation and decreased levels of synaptophysin in the brains of the mice. Several studies have thus identified both oxidative stress and Cu dyshomeostasis in AD, indicating possible causal mechanisms.

There have also been a number of *in vivo* and *in vitro* studies showing a link between Cu, APP, and Aβ. Researchers have used human fibroblasts over-expressing the Cu efflux protein, the Menkes protein (MNK), in order to test the theory that Cu may regulate APP expression (Bellingham et al., 2004). MNK deletion fibroblasts show high levels of intracellular Cu, while fibroblasts that overexpress MNK have depleted intracellular Cu (2004). APP gene expression was down-regulated in Cu depleted cells, where there would be an excess of extracellular Cu, due to the over activity of the efflux protein (2004). In studies of Tg mice, Cu levels have been found to be elevated in APP knock-out mice while Cu levels in mice over-expressing APP are reduced (White et al.,

1999; Maynard et al., 2002). Increases in brain Cu levels decrease the amyloidogenic pathway of AD, by increasing the likelihood of APP cleavage that favors the harmless nonamyloidogenic pathway (Borchardt et al., 1999). However, Cu has a high binding affinity for Aβ after it has formed, resulting in the formation of free radicals (Rivera-Mancía, Pérez-Neri, Ríos, Tristán-López, Rivera-Espinosa & Montes, 2010). This indicates a complex relationship between Cu and Aβ, as to Cu being preventative in Aβ formation, or being more harmful once the Aβ is present; thus, there is *in vitro* and *in vivo* evidence for both Cu deficiency and Cu toxicity in AD.

Returning to Cu's relationship to Zn, it has been suggested that the memory deficits seen in animals receiving excess Zn occurs through a Zn induced Cu deficiency, as Zn and Cu compete for entrance into the body, influencing the bioavailability of both metals (Maret & Sandstead, 2006; Klevay, 2008). It has been documented that high levels of Zn intake relative to Cu can cause a decline in Cu-dependent enzymes, i.e. SOD1, ceruloplasmin (major copper-carrying protein in the blood) and cytochrome c oxidase (found in mitochondria which direct cellular energy), as well as induce changes in immunological parameters, cholesterol, and lipoproteins (Maret & Sandstead, 2006). These particular aspects of Cu deficiency, which can be caused by high Zn intake, are significant, as they support various other theories concerning the etiology of AD (neuroinflammation, oxidative stress, and energy depletion). The behavioral data mentioned, in which Zn induced cognitive deficits were remediated by Cu supplementation, also alludes to this idea of a link to Cu deficiency and AD; however,

there is a necessity to test the theory directly through the use of a Cu deficiency using a diet with altered Cu content.

In order to observe the aforementioned cognitive deficits apparent in AD in conjunction with the added metal component, we used novel object recognition and Morris water maze tasks to assess declarative and spatial memory deficits, respectively, that are seen early in AD. NOR may serve as a measure of both episodic and semantic memory (types of declarative memory), as the mouse must remember that it has seen its standard object (episodic, dependent on time and space), but it may only need to recall attributes of the object, i.e. shape and color, which are not dependent on time and space (semantic). MWM was used to assess spatial memory, which relies on the hippocampus, a part of the medial temporal lobe impaired early in AD patients (Braak & Braak, 1991).

Overall, this research sought to further understand the relationship between Zn, Cu, and AD through the development of two experimental diets, differing only in Cu content. One diet was made in order to identify the effects of Cu deficiency in an early onset mouse model of AD, while the other was made as a control. Mice were raised in one of three experimental conditions; Cu control diet + lab water, Cu deficient diet + lab water, Cu control diet + Zn water, and tested in two behavioral tasks, novel object recognition (NOR) and Morris water maze (MWM) beginning at approximately 6 months of age.

CHAPTER TWO: MATERIALS AND METHODS

Mice:

Subjects consisted of Tg, J20, mice with a single copy of a doubly mutated hAPP transgene (Swedish: K670N/M671L and the Indiana: V717F mutations, using the human platelet derived growth factor, B polypeptide (PDGFB) promoter) and Wt mice of C57BL/6J (B6) background. The Tg mice chosen for this experiment were originally developed by Dr. Lennart Mucke, the Gladstone Institute, UCSF. This strain is similar to those previously used in our lab, CRND8. J20 and CRND8 mice both express doubly mutated forms of hAPP; the Swedish (K670N/M671L) and the Indiana (V717F) mutations; however, the CRND8 mouse uses a hybrid background strain that the J20 does not. The background strain used for the J20 is the most commonly used in laboratory animal research, the C57Bl/6J, which is the same strain we used as our Wt breeders. J20 mice develop diffuse amyloid plaques by age 5-7 months and behavioral deficits by 4-7 months. Mice also exhibit high rates (>15%) of premature death by 6 months.

All breeding animals were obtained from the Jackson Laboratory; experimental animals were bred at George Mason University. Harem breeding was utilized: one male J20 was paired with up to 3 female B6 mice for a period of 14 days. Females were then separated and singly housed in preparation for giving birth. At postnatal day 11-21, a small tail snip was collected from the pups for genotyping (Transnetyx, Cordova, TN).

Pups were weaned at postnatal day 21-30 according to sex and genotype (hAPP or Wt) with 2-4 mice per cage. Single housing was avoided whenever possible. Each cage contained two igloos, one with a wheel attachment. Experimental animals were handled 3-4 times per week. Mice were housed in a climate controlled room held at 22°C ± 3°C and 45-65% humidity with a 12:12 light/dark schedule and *ad libitum* access to food and water. All animals were cared for and experiments completed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and George Mason University Institutional Animal Care and Use Committee (IACUC) Guidelines.

Food:

All animals received food and water *ad libitum*. Breeder animals were fed a standardized laboratory diet (Harlan T.7012). Two experimental diets were created with Harlan nutritionists for the experimental animals, i.e. “Cu deficient” and “Cu control”. Experimental mice were transitioned to the Cu control diet upon weaning and their group specific diets at 8 weeks of age, when they were also put on their experimental water. The lag in transition time was to account for the immunocompromised state of the Tg animals, giving them more time to acclimate to their new environment before being placed on a potentially harmful diet.

The new diets were created using the Zn and Cu levels in 7012 as a guideline, attempting to keep the Zn levels as close to our standard diet as possible, so as to remain in accordance with previous studies (7012= ~63ppm Zn, new diet= 40ppm Zn) (Railey et al., 2010; Railey et al., 2011). The T.7012 diet contains non-nutritive substances, such as phytates, which affects the absorption of minerals; because of this, minerals in the 7012

diet are generally higher than what we would need for the purified diet created for this experiment (T. Herfel PhD., Harlan industries, personal communication, March 12, 2013). Both diets are identical except for the Cu content (control= 16ppm Cu, deficient= 4ppm Cu). Additionally, the Cu control diet had the same Zn/Cu ratio as T.7012; however, the Zn/Cu ratio was much higher in the Cu deficient diet as a result of the decreased level of Cu with a constant level of Zn.

Water:

Mice were transitioned to their experimental water conditions on the same day as their hard diet, at 8 weeks of age. A subset of animals was transitioned to lab water with the addition of 10ppm Zn carbonate. Animals not receiving enhanced Zn were given laboratory tap water. Zn water was prepared using a 10,000ppm solution of Zn dissolved in 5% nitric acid. The solution was buffered using sodium carbonate ($NaCO_3$) to bring it to a pH of 7. Methods for preparation were in accordance with previous studies (Chrosniak et al., 2006; Linkous, et al., 2009; Railey et al., 2010; Railey et al., 2011). Water samples were taken and routinely tested for metal content using inductively coupled plasmaoptical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS, Reston,VA).

All animals remained on their group specific diets/water from 8 weeks of age until sacrifice. Sacrificing occurred following behavioral testing at approximately 6.5 months of age, after 4.5 months of dosing.

Behavioral Testing:

Experiment 1: Novel Object Recognition (NOR):

NOR testing began at approximately 6 months of age. Two animals were tested at a time in separate testing boxes. The boxes were 4 sided, blue Plexiglas 18 inch by 18 inch and 9.5 inch tall squares (Clever Sys., Inc., Reston, VA). Objects used were of similar size to the mice and were attached to the bottom of the boxes with Velcro. Four different objects were used. A video camera mounted directly above the boxes in the testing room recorded every trial. Sniffing behavior analysis was conducted using TopScan (Clever Sys., Inc.), and was measured as nose sniffing when the mouse's nose was directly facing the object. Animals were ran in random order and counterbalanced for genotype, diet, standard object, box, and standard object location. Testing was assessed over 4 days. The protocol used was similar to previous studies with minor alterations (Bevins & Besheer, 2006; Yuede et al., 2009).

Habituation: Animals were habituated for two days, at which time they were individually placed in their designated testing box for 10 minutes with no objects present. Habituation was conducted to familiarize the mice with the testing environment to avoid stress and environment novelty as confounding variables.

Object Recognition: Object recognition was assessed over two days. Day 1 consisted of three trials. Trial 1 was their initial exposure to their standard object. Each mouse was placed in their designated testing box with 2 copies of their standard object for 10 minutes. Trial 2 occurred 15-minutes after initial exposure to the standard object and lasted 4 minutes. During testing trials, mice were placed in the testing box with their

standard object (standard objects appeared in the same location for every testing trial) and one "novel" object that they had not been exposed to. Trial 3 was carried out exactly as Trial 2, 1-hour after initial exposure and with a new "novel" object. Testing Day 2 was a long term, 24-hour retention trial. Mice were again placed in their testing box with their standard object and a new "novel" object for 4 minutes. Between trials, the testing boxes and objects were cleaned with 70% ethanol.

Table 1. Schedule of Novel Object Recognition Days

Day	Retention Interval	Objects	Time in Box
1	Habituation	None	10 minutes
2	Habituation	None	10 minutes
3	Initial	Standard + Standard	10 minutes
3	15-minutes	Standard + Novel 1	4 minutes
3	1-hour	Standard + Novel 2	4 minutes
4	24-hours	Standard + Novel 3	4 minutes

Table 2. Groups of tested Animals: NOR

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=15	n=17	n=16	n=48
Tg	n=14	n=13	n=13	n=40
Total	n=29	n=30	n=29	n=88

Experiment 2: Morris Water Maze (MWM)

MWM was conducted four days post NOR, when all mice were just under 6.5 months old. Mice were placed in a 4ft diameter pool with a hidden, transparent, Plexiglas platform submerged approximately 5mm below the surface of the water. Water was dyed white with non-toxic tempura paint (Becker's School Supplies, Pennsauken, NJ) to

further hide the platform. The pool was divided into 4 quadrants by visual cues on posts approximately 12 inches from the pool's edge. Cues were large black and white cut out shapes. Water temperature was maintained at 24°C ± 2°C. Testing occurred over 8 days, with 6 testing days, 1 24-hour probe trial, and 1 day of visual platform at the end of the testing paradigm. Testing days each consisted of 3 trials of up to 60 seconds with 45-second inter-trial intervals. Trial start locations were the same for each mouse within a day; start locations varied across days. The hidden platform remained in the same quadrant for all days except for visual platform. Recording and analysis was conducted using Coulbourn Instruments WaterMaze3 (Allentown, PA) software and computerized tracking system.

Behavioral measures used were latency to platform, platform crossings on probe trials, percent time spent in target quadrant on probe trials, and thigmotaxia. Thigmotaxia is used as a measure of anxiety-like behavior, and is defined as the amount of time (percent) spent swimming the outermost 10% of the pool, closest to the edge. Thigmotaxia may also be measuring learning/search strategy.

Atlantis Platform: On testing days 2,4, and 6, trial 3 consisted of a "probe trial". Day 7 consisted of only one probe trial as a measure of long term, 24-hour, spatial reference memory. During these trials the platform was completely submerged while the animals swam for 60 seconds. After 60 seconds the platform was raised, and the animals were gently guided to the platform where they were allowed to sit on the platform for 15 seconds to observe their surroundings. During these trials we were most interested in the

number of times the mouse crossed the location where the platform should have been, i.e. platform crossings and the % of total time spent in the target quadrant.

Visible Platform: On day 8, the original platform was removed and a separate, larger platform was placed in a different quadrant. The new platform was equipped with a tower that had alternating black and white stripes, making the platform location clearly visible to the mice. These trials were used to identify any mice with sensory or motor deficits. Mice were given two trials, up to 60 seconds, that were 45 seconds apart.

Table 3. Schedule of Morris Water Maze Days

Day	Number of Trials	Platform Location	Max. Trial Length	Intertrial interval
1	A,B,C	Stationary (submerged 5mm)	60 seconds	45 seconds
2	A B **C: Probe 1**	Stationary (submerged 5mm) Stationary (submerged 5mm) **Atlantis (Platform Unavailable)**	60 seconds 60 seconds 60 seconds	45 seconds
3	A,B,C	Stationary (submerged 5mm)	60 seconds	45 seconds
4	A B **C: Probe 2**	Stationary (submerged 5mm) Stationary (submerged 5mm) **Atlantis (Platform Unavailable)**	60 seconds 60 seconds 60 seconds	45 seconds
5	A,B,C	Stationary (submerged 5mm)	60 seconds	45 seconds
6	A B **C: Probe 3**	Stationary (submerged 5mm) Stationary (submerged 5mm) **Atlantis (Platform Unavailable)**	60 seconds 60 seconds 60 seconds	45 seconds
7	**A: 24hr Probe**	**Atlantis (Platform Unavailable)**	60 seconds	N/A
8	A,B	Visible (above 5mm)	60 seconds	45 seconds

Table 4. Groups of tested Animals: MWM

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=15	n=17	n=16	n=48
Tg	n=13	n=13	n=12	n=38
Total	n=28	n=30	n=28	n=86

CHAPTER THREE: RESULTS

Experiment 1: Novel Object Recognition (NOR):

Using the Huynh-Feldt correction, a 2x3x3 (Genotype x Diet x Retention Interval) mixed design ANOVA on duration of object sniffing showed a trend towards a significant effect of retention interval, $F(1.848,151.573)=3.002$, $p=.057$. There was no significant between-subjects effect of genotype (Figure 1). All mice displayed evidence of object recognition at 15-minutes, varied greatly at 1-hour, and Tg mice showed no object preference at 24-hours. A series of one-sample t-tests, using 50% (.5) as the test value for object recognition, were conducted to further analyze the 1-hour and 24-hour retention interval for differences in diet. The p values were adjusted to reflect the number of tests being ran ($p<.05$ to $p<.004$).

At 1-hour, Tg Cu controls maintained novel object recognition ($t=4.555$, $p<.001$), while Tg Cu deficient and Zn-enhanced mice no longer showed a preference for their novel objects ($t=.296$, $p>.05$; $t=1.584$, $p>.05$) (Figure 2C). No Tg groups showed novel object preference at 24-hours.

Wt mice showed differing trends than Tg mice at 1-hour: Cu deficient mice maintained novel object preference ($t=3.463$, $p=.003$), while ZN-enhanced and Cu control groups did not ($t=1.136$, $p>.05$) (Figure 2B). Wt groups did not show novel object preference at 24-hours.

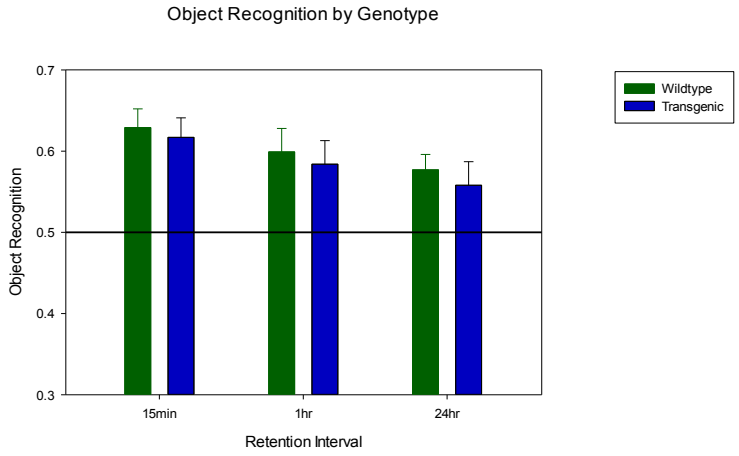


Figure 1. Object Recognition by Genotype.
There was no significant between-subjects effect of genotype ($p>.05$).

Table 5. Significant Novel Object Preference by Genotype, Retention Interval and Diet

	15min	1-hour	24-hours
Wt	All Groups	Cu Deficient	None
Tg	All Groups	Cu Control	None

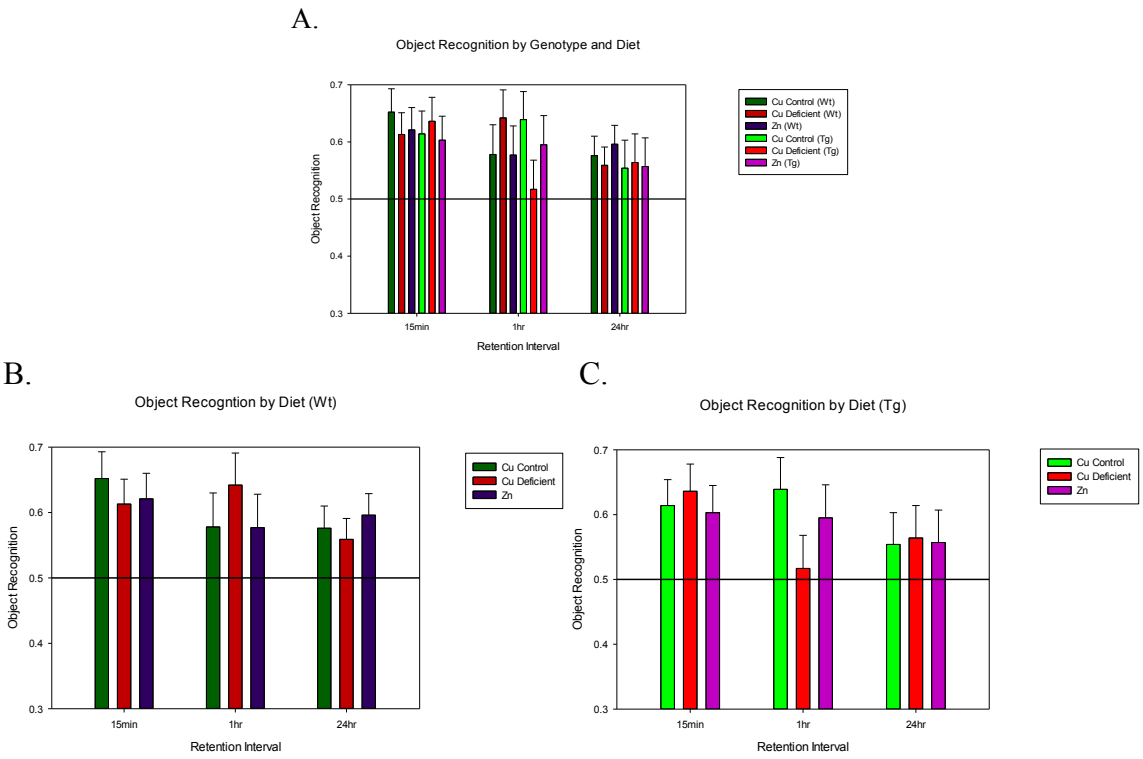


Figure 2. Object Recognition by Genotype and Diet.
No overall diet effect was seen. Wt Cu deficient mice show novel object preference significantly higher than 50% at 1-hour (p=.003), while Cu control and Zn-enhanced Wt mice do not. Tg Cu control mice maintain novel object recognition above 50% at 1-hour (p<.001), while Tg Cu deficient and Zn-enhanced mice do not. No object preference was seen at 24-hours in Wt or Tg mice.

Experiment 2: Morris Water Maze (MWM):

A 2x3x6 (Genotype x Diet x Day) mixed ANOVA on latency to the escape platform, showed a significant difference between Wt and Tg mice across days, with Wt mice showing consistently faster escape latencies, F(1,80)=20.031, p<.001 (Figure 3).

There was also a significant main effect of day, indicating that the mice did learn the task as days progressed, $F(5,400)=13.749$, $p<.001$. Examination of days 2-5 showed a significant interaction between day and diet, $F(6,240)=2.113$, $p<.05$. Further analysis showed that the interaction was being driven by a day x diet interaction for the Tg mice, $F(6,105)=2.236$, $p<.05$ (Figure 4). Pairwise comparisons showed a marginally significant difference between Tg Cu deficient and Tg Zn mice on day 3 latency, $p=.059$; Tg Zn-enhanced mice did not learn the task until day 4, (Figure 4C). Wt and Tg mice dosed on Zn were significantly different on day 3, with Wt mice having a faster latency $p<.01$ (Figure 5C). Wt and Tg Cu deficient, as well as Wt and Tg Cu controls, showed a significant difference in latency on day 2, $p<.05$ (Figure 5A and 5B). In general, for the Tg mice, Zn-enhanced mice performed the worst, Cu controls performed intermediately, and Cu deficient mice performed the best. Interestingly a 2x2x6 (Genotype x Sex x Day) mixed ANOVA showed a main effect of sex in the Wt mice, $F(1,46)=8.944$, $p=.004$, with female mice showing significantly longer latencies to find the escape platform.

A 2x3x4 (Genotype x Diet x Day) mixed ANOVA on percent of time spent in the target quadrant, showed a significant between-subjects effect of genotype, with Wt mice spending more time in the correct quadrant than Tg's, $F(1,80)=3.969$, $p<.05$. Using the Huynh-Feldt correction, there was a significant main effect of day, $F(6,480)=7.603$, $p<.01$ (Figure 6A). Although non-significant, data suggests a trend for a day by diet interaction ($F(5.743,116.293)=1.647$, $p=.138$) for the transgenic mice ($F(5.839,77.512)=3.032$, $p=.131$) (Figure 6A). No overall effect of diet was seen on this measure (Figure 6B). A 2x3x4 (Genotype x Diet x Day) mixed ANOVA on the number

of platform crossings on probe trials, showed a significant between-subjects effect of genotype, with Wt mice crossing the platform location significantly more than Tg mice, $F(1,80)=18.760$, $p<.001$ (Figure 7A). There was also a significant main effect of day, $F(3,240)=7.520$, $p<.001$ (Figure 7A). Again, no overall effect of diet was seen (Figure 7B).

A 2x3x7 (Genotype x Diet x Day) mixed ANOVA on thigmotaxia, showed a significant between subjects effect of genotype, with Wt mice spending significantly less time near the edge of the pool, $F(1,80)=31.905$, $p<.001$ (Figure 8A). Using the Huynh-Feldt correction, there was a significant main effect of day, with thigmotaxia decreasing as the days progressed, $F(5.265,59.385)=56.590$, $p<.001$, and a significant interaction between day and diet on days 3-6, $F(5.501,220.037)=81.801$, $p<.05$ (Figure 8B). Interestingly, the effect of diet was seen in the Wt group. Pairwise comparisons show that Wt Cu control mice exhibit increased anxiety-like behavior compared to Cu deficient mice on day 4, $p=.058$, and significantly more than Cu deficient and Zn-enhanced mice on day 5, $p<.05$ (Figure 8B). This indicates that, while Cu deficient and Zn-enhanced wild type mice exhibit decreased anxiety-like behavior beginning on day3, Cu control mice do not show a reduction until day 6.

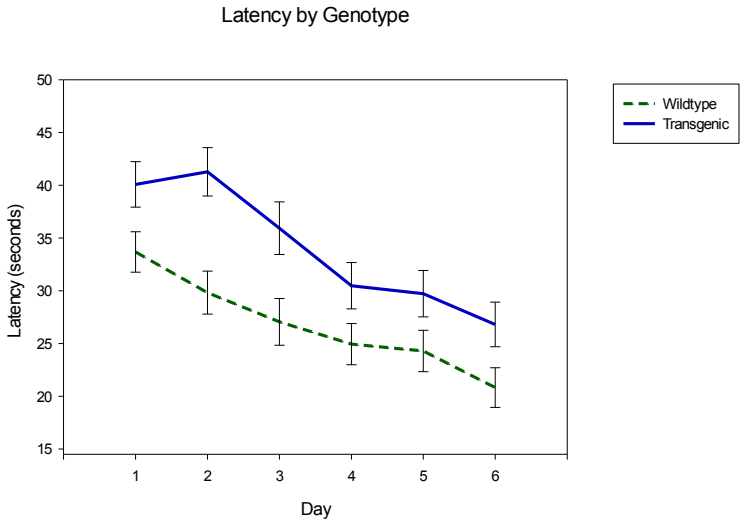
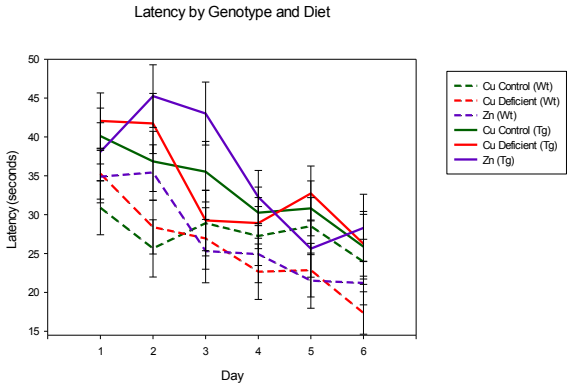
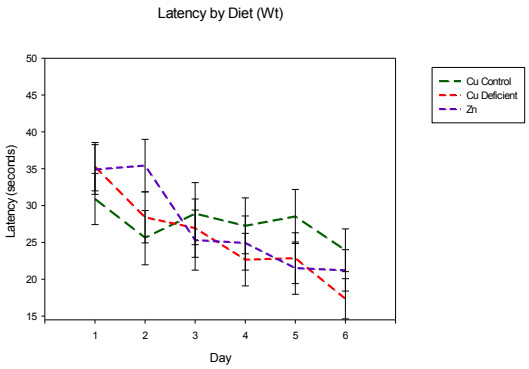


Figure 3. Average Latency by Genotype.
Wt mice exhibited significantly faster average latencies across days 1,2,3, and 6 ($p<.05$). There was a marginally significant difference on days 4 ($p=.063$) and 5 ($p=.070$).

A.



B.



C.

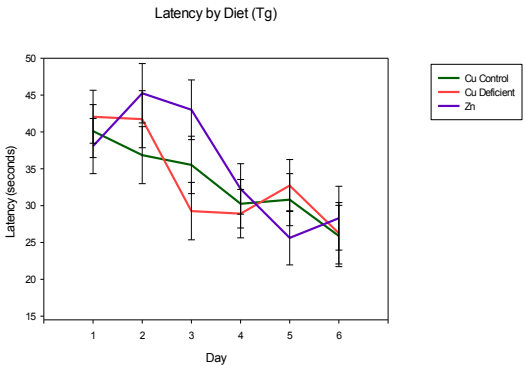
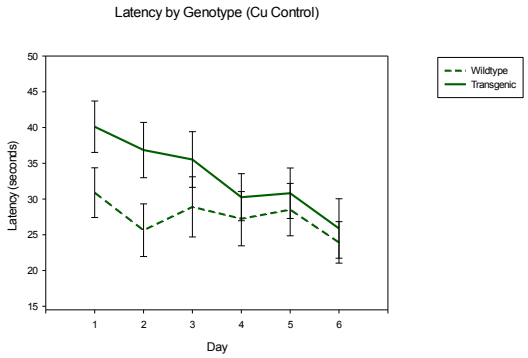
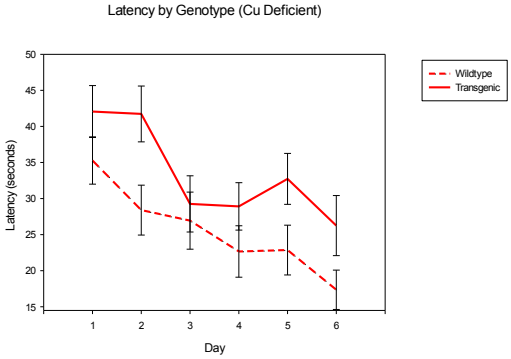


Figure 4. Average Latency by Genotype and Diet.
Days 2-5 showed a significant interaction between day and diet ($p<.05$), driven by an interaction in the Tg mice ($p<.05$). Pairwise comparisons showed a marginally significant difference between the Cu deficient and Zn-enhanced groups on day 3, indicating a delay in learning for the Zn-enhanced mice ($p=.059$).

A.



B.



C.

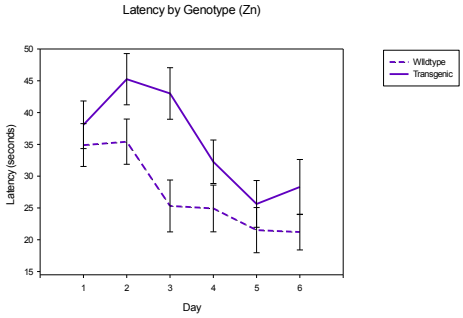
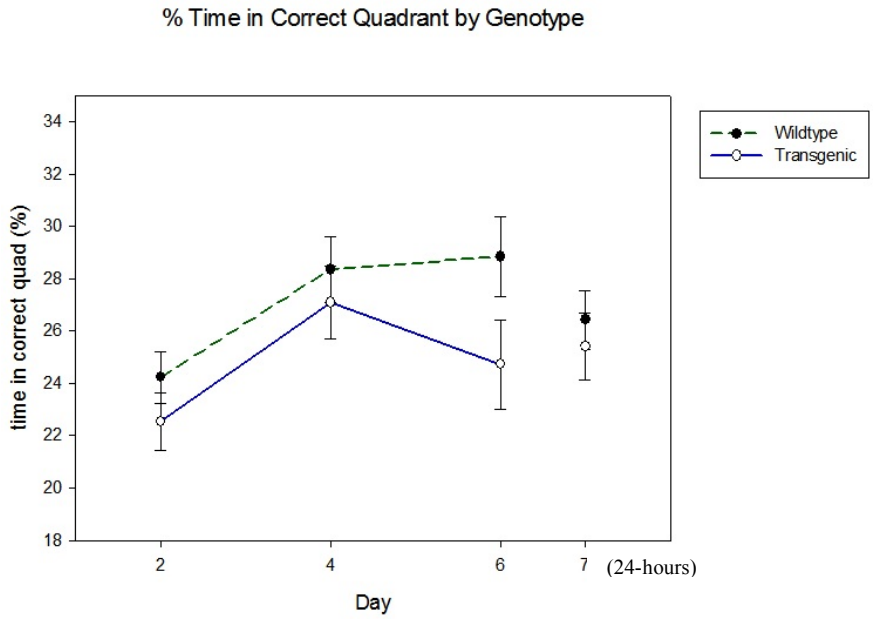


Figure 5. Average Latency by Diet.
A. Pairwise comparisons showed a significant difference between Wt and Tg Cu control mice on day 2 ($p<.05$). **B.** Pairwise comparisons showed a significant difference between Wt and Tg Cu deficient mice on day 2 ($p<.05$). **C.** Pairwise comparisons showed a significant difference between Wt and Tg Zn-enhanced mice on day 3 ($p<.01$).

A.



B.

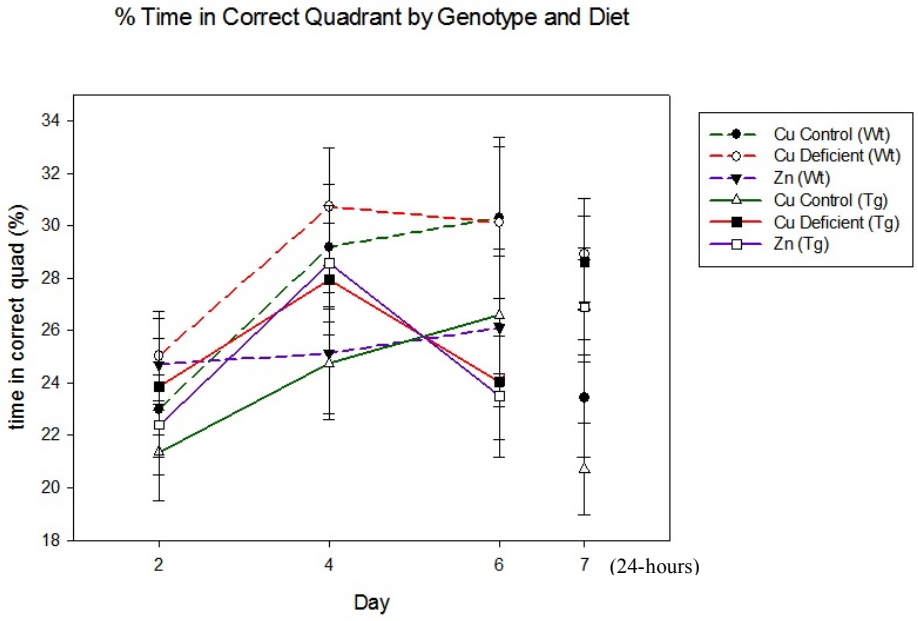
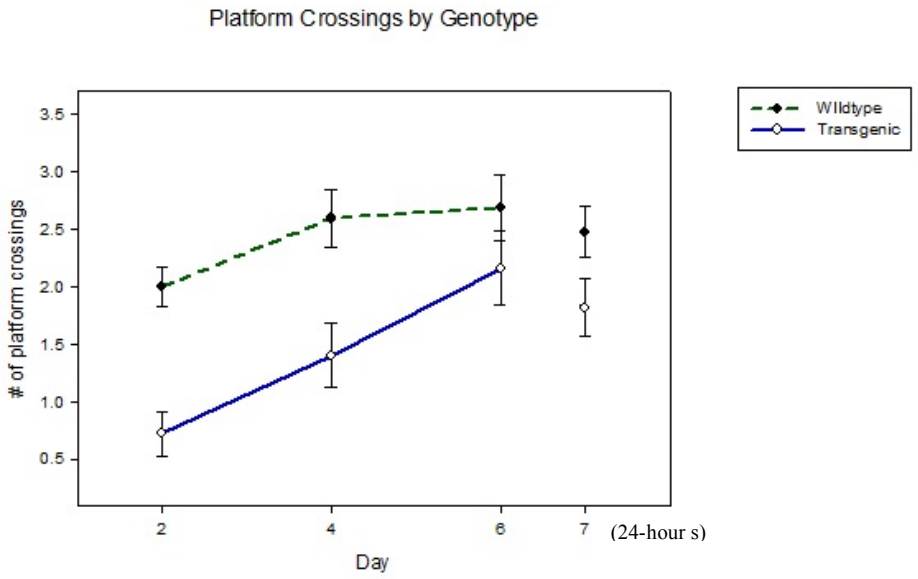


Figure 6. Time spent in Correct Quadrant (%).
A. There was a significant between-subjects (Genotype) effect for % time spent in the correct quadrant, ($p<.05$). There was also a trend towards a significant interaction between day and diet in the Tg mice, ($p=.131$). **B**. No overall effect of diet was seen.

A.



B.

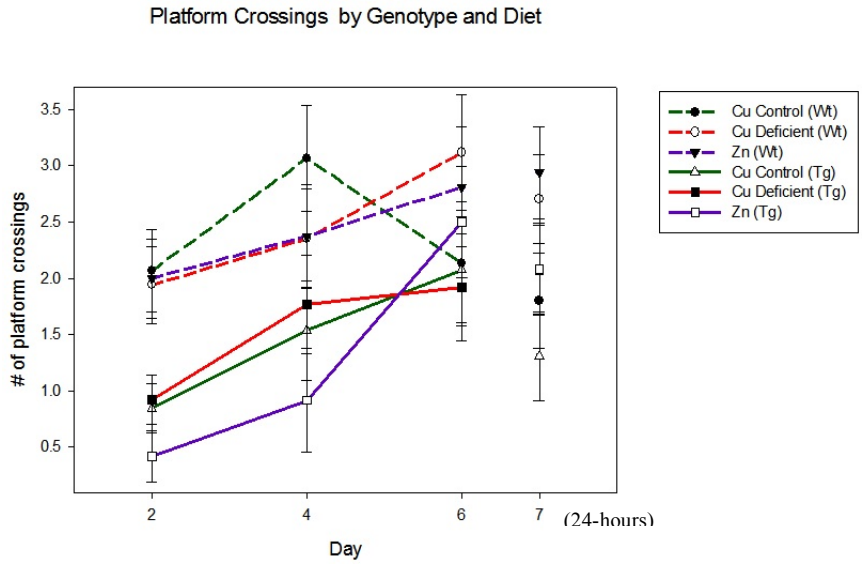
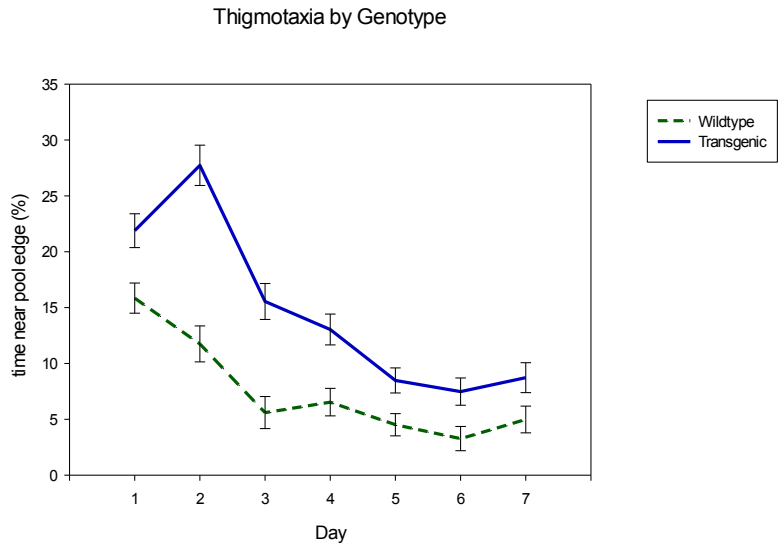


Figure 7. Number of Platform Crossings.
A. Wt mice showed significantly more platform crossings than Tg's across days ($p<.001$). **B**. There was also a significant main effect of day, with the number of platform crossings increasing as days progressed ($p<.001$). No overall effect of diet was seen.

A.



B.

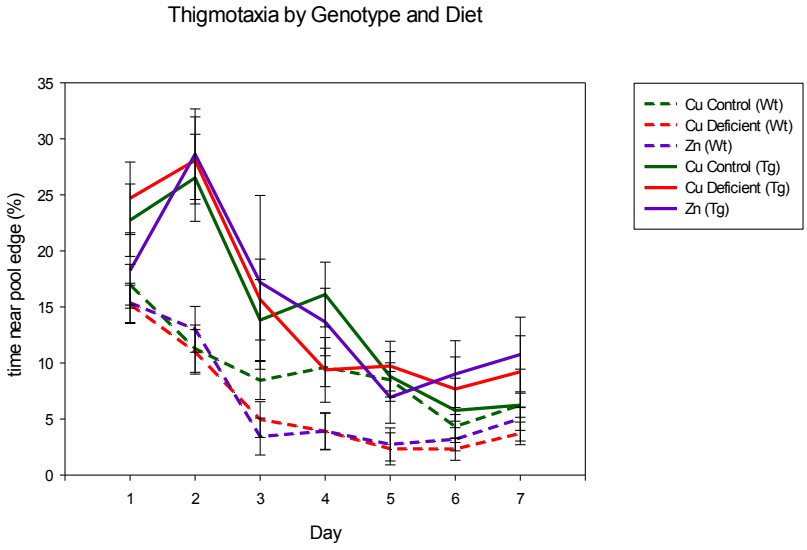


Figure 8. Time Spent Near Pool Edge (%) (Thigmotaxia).
A. Wt mice spent significantly less time near the outermost 10% of the pool than Tg's ($p<.001$). Thigmotaxia significantly decreased as days progressed ($p<.001$). **B**. There was a significant interaction between day and diet on days 3-6 ($p<.05$). Pairwise comparisons show that Wt Cu control mice exhibit increased anxiety-like behavior compared to Cu deficient mice on day 4 ($p=.058$) and significantly more than Cu deficient and Zn-enhanced mice on day 5 ($p<.05$).

CHAPTER FOUR: DISCUSSION

The goal of this study was to further examine the roles of Zn and Cu in AD phenotypic progression. The role of Zn in AD seems to be better established, while studies involving Cu are less clear. Studies on increased Cu in high cholesterol diets indicate cognitive dysfunction in a rabbit model of AD (Sparks & Schreurs, 2003). There is also some recent evidence that even low-level increases in Cu may negatively alter production and clearance of Aβ (Singh et al., 2013). In contrast, other studies have found that behavioral deficits induced by administration of excess Zn could be remediated by the addition of Cu (Chrosniak et al. 2006; Railey et al., 2010; Railey et al., 2011). These results suggest that the excess Zn may be causing a Cu deficiency, thus we looked at a direct Cu deficiency achieved through dietary means.

NOR results suggest that Cu deficiency can affect longer-term declarative memory in AD. In the Tg group, Cu deficient mice showed novel object preference at 15-minutes, but not at 1-hour. There were no significant differences between diet conditions; however, when viewed from a perspective of mean difference from chance (.5 or 50%), the diet differences are clearer. Cu control Tg mice maintain object recognition, even through the 1-hour time interval, while Cu deficient and Zn-enhanced groups only show a significant novel object preference at the short-term, 15-minute measure. Overall, Tg mice show a general capacity for short-term memory, but exhibit group specific

disruptions in longer-term memory in both of the experimental conditions. One possible explanation for the lack of significant differences between the diet groups, despite some substantial mean differences between the Cu control and Cu deficient mice at 1-hour, could be attributed to a large amount of variability in the sample.

Unlike NOR, MWM results suggest a longer-term benefit of mild Cu deficiency in AD, as the Tg Cu deficient group consistently performed the best throughout the task. They learned the task faster than the Tg Zn group, showing a mean difference on day 3 latency that is marginally significant, p=.059. These results are consistent with hypotheses by Harris et al., in which they postulated that reduction of brain Cu, which both promotes Aβ aggregation and generates reactive oxygen species, would be a viable strategy to regulate the formation of insoluble Aβ in Tg mice (Tg2576) (2014). In this study, they induced the Cu deficiency through Zn acetate supplementation in the subjects' drinking water, thereby reducing the absorption capacity of Cu from the diet. Although their behavioral data did not indicate a protective effect of Cu deficiency, biochemical assays did show a reduction of Aβ as well as a reduction in brain Cu (2014). For the current study, all between-subjects (Genotype) measures for MWM were significant.

While we expected the Zn-enhanced and Cu deficient mice to perform similarly on behavioral tasks (if the Zn effect was purely inducing a Cu deficiency), this was clearly not the case. These two groups showed the most substantial differences in MWM, at least in the Tg mice, indicating that 1.) the effects of Zn and Cu may be dependent on different brain structures, as evidenced by the differing behavior seen in NOR and

MWM, and 2.) the Zn effect is not entirely due to an induced Cu deficiency. NOR and MWM rely on different brain regions (entorhinal and perirhinal cortices vs hippocampus respectively). The metals may be localized differently or be found in varying concentrations in each region, which may explain the differences in behavior for the two tasks. Corona et al. (2010) also found differences in the effects of metal supplementation in MWM but not in NOR; however, a different mouse strain, that also contained Tau (3x mouse), was used, and the results of enhanced Zn were contradictory to ours, in that enhanced Zn showed positive behavioral effects (2010). Histological analyses will be conducted on the brains of selected mice from the current study in order to further investigate explanations for the differing behavioral results. Such variables as metal and plaque load, as well as levels of ZnT3 and some inflammatory cytokines will be looked at.

Overall, this study indicates that there is an interaction between metals in the daily diet that depends on the presence or absence of disease, evidenced by the differing trends seen in the Wt and Tg mice. In contrast to previous studies, we did not see a significant difference between the Tg control and Tg Zn groups; however, the administration of Zn did clearly cause impairments, especially in MWM. Many previous studies that showed Zn deficits used a Harlan laboratory standard diet T.7012, which differed in metal levels, non-nutritive substances, and soy, from the diets used for this study. T.7012 has a Zn level of approximately 60ppm and Cu content of 20ppm; the newly developed purified control diet had levels of 40ppm and 16ppm (Cu deficient=4ppm of Cu) respectively. It is possible that the previously established 10ppm Zn addition was not high enough to

achieve a peak concentration to show significant differences from controls. Another consideration is that the newly developed diets were purified, with more controlled levels of ingredients. For example, T.7012 contains non-nutritive substances, such as phytates, that may affect how much of the metals are actually absorbed into the system. Lastly, the newly developed diets also contained soybean oil, a source of soy, which T.7012 does not; research shows that soy increases levels of estrogen. Additionally, isoflavones found in soy can bind to estrogen receptors and possibly effect behavior, especially when gender is considered; this may have contributed to the differing behavior in Wt male and female latency in MWM. A study by Simpkins et al. showed a reversal of learning and memory impairments in Sprague-Dawley rats with estrogen replacement therapy (ERT) (impairments induced by ovariectomy) (1997). They also found that ovariectomies caused a decrease in cholinergic uptake and choline acetyltransferase, both of which are implicated in AD. However, the effects seen in this study cannot necessarily be attributed to increased soy, and thus increased estrogen binding, as the female Wt mice took significantly longer to find the escape platform than did male Wt's. The current study's Wt gender difference is consistent with another study in our lab; Wt males showed significantly faster latencies than female Wt's, and no gender difference was seen in the Tg mice (Groeber, 2013). It is possible that AD pathology caused such strong behavioral impairments that any gender differences may have been obscured in the Tg mice. While many aspects of the diets are different, the experimental control diet and the previously used T.7012 have similar Zn/Cu ratios (2.5 and 2.7 respectively). The Zn/Cu ratio for the Cu deficient diet was substantially different (10); however, this was a

variable that could not be controlled for, due to our desire to keep all levels of ingredients the same between the diets, apart from the Cu.

Results from this study indicate that cognitive deficits are affected by daily diet and the balance of physiologically important metals. When using AD Tg mouse models, it may be more important than previously thought to control for the type of diet, in favor of diets that allow for tighter control over what is actually being absorbed, the ratio of specific ingredients, and the diet base, such as one that is soy-based versus one that is not. This difference in daily diet may have a large part in the contradictory results seen in the field, as a number of different laboratory rodent diets exist. Therefore, in order to answer the question of how metals are affecting the AD brain, we must work to identify specific dietary interactions, i.e. metal interactions with diet ingredients, such as soy, phytates, and phytoestrogens, as well as the metal levels themselves.

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BIOGRAPHY

Stefanie Howell was born in Dearborn, Michigan to Cheryl and Kim Toms. She attended grade school in the Van Buren Public School District. After graduating from Belleville High School in 2006, she attended Eastern Michigan University (Ypsilanti, MI) for 2 years, before moving to Okinawa, Japan with her husband, Richard Howell. She finished her Bachelor of Science degree, dual majoring in Psychology and History, from the University of Maryland University College while in Okinawa in 2010. Stefanie started graduate school in the Fall of 2011 after giving birth to her first daughter, Aviana Jean Howell. This thesis serves as completion of her Master of Art's degree, concentration in Cognitive and Behavioral Neuroscience, at George Mason University, Fairfax, VA.