A LONGITUDINAL INVESTIGATION INTO THE EFFECT OF ZN AND CU ON SPATIAL LEARNING/MEMORY AND SOCIAL PREFERENCE IN A MOUSE MODEL OF LOAD

by

Stefanie N. Howell

A Dissertation
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of
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Psychology

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Date: _____ Fall Semester 2016
George Mason University
Fairfax, VA

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By

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DEDICATION

This dissertation is dedicated to my children, who constantly remind me to cherish every day.

ACKNOWLEDGEMENTS

First, I would like to thank my committee members, Dr. Jane Flinn, Dr. Craig McDonald, and Dr. Jennifer Brielmaier-Sontag for their support and guidance for the duration of my doctoral degree. I would also like to thank my family and friends for being a constant and unwavering support system during such a strenuous journey. Lastly, but certainly not least, I would like to thank all of the graduate and undergraduate students who assisted with this study; I could not have done it without your support.

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LIST OF ABBREVIATIONS AND SYMBOLS

Alzheimer's Disease……AD
Amyloid Beta……..……...Aβ
(Human) Amyloid Precursor Protein…….(h)APP
(Human) Apoliprotein E.....…….(h)ApoE
(Human) Amyloid Precursor Protein/Apoliprotein E4.....…..…..….(h)APP/(h)E4
Copper……...……Cu
Iron.....Fe
Morris Water Maze…….MWM
Olfactory Habituation/Dishabituation…….…...…OHD
Open Field.....…….....…OF
Transgenic……...…..Tg
Wildtype……...….Wt
Zinc……Zn

ABSTRACT

A LONGITUDINAL INVESTIGATION INTO THE EFFECT OF ZN AND CU ON SPATIAL LEARNING/MEMORY AND SOCIAL PREFERENCE IN A MOUSE MODEL OF LOAD

Stefanie N. Howell, PhD

George Mason University, 2016

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This study examined the effects of dietary copper (Cu) and zinc (Zn) on spatial learning/memory and social preference in a mouse model of late onset Alzheimer's disease (AD). Previous research has indicated that excess dietary Zn may cause behavioral impairments through an induced Cu deficiency. Using a mildly Cu deficient hard diet, we initially tested this theory in an early onset model of AD, which suggested instead that 1.) the effects of Cu and Zn may differ in the brain regions controlling different behaviors, and that 2.) the effect of excess Zn may not be entirely due to an induced Cu deficiency.

To examine the effects of the dietary manipulations in a late onset model, we crossed male hAPP transgenic (Tg) mice with homozygous ApoE4 females. We were particularly interested in the E4 allele of the APOE gene, as it constitutes a high risk factor for development of AD, with some evidence showing that it may be protective

early on. Six groups were tested for spatial learning/memory (Morris water maze (MWM)) and social preference (3-chamber apparatus) at 6 and 12 months of age (wildtype (Wt) and Tg mice on (a.) lab water + Cu control diet, (b.) lab water + Cu deficient diet, or (c.) Zn-enhanced water + Cu control diet). These time points were chosen to identify longitudinal changes in behavior, allowing for identification of the potentially protective effects of the E4 allele and dietary interactions in early and late stages of AD. Open field (OF) and olfactory habituation/dishabituation (OHD) were assessed as controls for the social behavior task (locomotor activity and odor sensitivity respectively).

Data at 6 months suggests early spatial learning/memory deficits, but intact social preference in AD mice. AD mice showed significantly longer latencies, fewer platform crossings, and spent less time in the target quadrant than Wt's in the MWM ($p<.001$), but display normal preferences for social interaction in the 3-chamber test. Tg Cu deficient and Zn mice display a normal preference for social novelty, indicating that excess Zn, whether direct or indirect, may uniquely improve behavior on this task, as the LOAD mice are expected to be Zn deficient. At 12 months, all mice still exhibit normal social interaction during trial 2 of the pro-social task, but no significant differences were seen for trial 3. There remains a significant difference between genotypes in measures of MWM, but no significant effects of diet were indicated at either time point.

CHAPTER ONE: INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia, accounting for approximately 60-70% of all documented dementia cases (Mielke, Vemuri, & Rocca, 2014). Two forms have been identified, early onset (EOAD), generally occurring in those younger than 65, and late onset (LOAD), which affects those older than 65 years of age. EOAD is rare, documented in less than 5% of all AD cases, but is the most commonly studied in mouse models; the reliance on early onset models is a limitation of many AD studies. LOAD is the most common form; however, unlike EOAD that has been largely attributed to mutations on specific genes, LOAD has a mere genetic component (APOE gene, chromosome 19) that is heavily affected by lifestyle and environmental factors (diet, exercise, education, etc.), making it more difficult to study. In regards to environmental and dietary factors that may contribute to LOAD progression, current research has emphasized the role of biometals, such as zinc (Zn), copper (Cu), and iron (Fe), with mixed results. This study sought to further investigate the complex relationship between LOAD and environmental factors, by examining the effects of excess dietary Zn and mild Cu deficiency on social interaction and spatial learning and memory in a transgenic (Tg) mouse model.

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). 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 (Jonsson et al., 2013; Harris et al., 2010; Roberson et al., 2007). ApoE, the protein coded for by the APOE gene (the primary genetic component of LOAD), has been strongly implicated in the modulation, accumulation and clearance of Aβ (Wildsmith, Holley, Savage, Skerrett, & Landreth, 2013; Mahley et al., 2009; Tokuda et al., 2000). It is co-localized within AD plaques and exhibits isoform specific binding to Aβ, possibly facilitating Aβ 's clearance (Tokuda et al., 2000; Mahley & Rall, 2000). The apoE4 isoform has shown deficiencies in Aβ clearance when compared to E2 and E3, as mice expressing the various isoforms of APOE had differing levels of Aβ in the interstitial fluid (ISF) and hippocampus (E4>E3>E2) (Wildsmith et al., 2013). Evidence suggests that APOE may serve as a molecular chaperone for Aβ, with apoE4 showing the least efficiency in this area (Moir et al., 1999, Bush, 2000). Such a decreased efficiency may be the result of apoE4's increased susceptibility for cleavage, leaving it in a state that has a much lower binding affinity for apoE receptors [when it is bound to Aβ] (Xu, Finkelstein, & Adlard, 2014). In addition to its effects on APP and Aβ, apoE4 has also been indicated in the hyperphosphorylation of tau, perhaps through interactions with Zn (Flinn, Bozzelli, Adlard, & Railey, 2014). ApoE4 promotes the phosphorylation of the tau protein via the extracellular signal-regulated kinase (Erk), which may be promoted by Zn (Harris, Brecht, Xu, Mahley, & Huang, 2004). High levels of extracellular compared to intracellular levels of Zn have been shown to destabilize microtubules, leading to the

presumption that apoE4 and Zn work in concert to increase AD pathology (Craddock et al., 2012).

Zinc (Zn) and other transition metals, such as copper (Cu) and iron (Fe), have also been shown to bind to and aggregate Aβ, as well as affect its functioning, cleavage, and processing (Zn and Cu are focused on for the scope of this dissertation) (Moir et al., 1999; Xu et al., 2014). As one ages, these metals in brain parenchyma tend to increase, while in the case of AD brains, the effects are further exacerbated (Moir et al., 1999). Regulation of these metals in the body is critical, as they have been tied to a number of life-sustaining mechanisms. Zinc has been tied to immune system, protein, hormone, antioxidant, transcription and replication functions (Bitanihirwe, & Cunningham, 2009). Additionally, it has been linked to neuromodulation and plasticity, and is a required component of many essential proteins and enzymes (Bitanihirwe, & Cunningham, 2009; Que et al., 2008). Copper has been identified as a crucial element in energy metabolism, antioxidation, iron metabolism, neurotransmitter synthesis and other neuromodulatory functions. Related specifically to AD, evidence suggests that APP and Aβ may function in Cu detoxification and efflux (Bellingham et al., 2004). Disruption of Zn and Cu homeostasis may then lead to a breakdown in the functions that they are responsible for regulating, as evidenced by their association with a number of diseases, namely AD, Amyotrophic Lateral Sclerosis (ALS), Schizophrenia, epilepsy, Menke's, Wilson's Parkinson's and Huntington's disease. With life expectancies in the United States and elsewhere continuing to rise, there is an emphasis being placed on ways to increase "healthy" aging, which includes changes in diet and exercise. A majority of elderly

individuals regularly partake in dietary supplementation (Bailey, 2010); many of who have little education in regards to the potentially harmful effects of too much supplementation. Excess amounts of supplementation are particularly concerning for those individuals with neurodegenerative disorders, as it may result in a more rapid expression and/or progression of those disorders (including biometal supplementation). In addition to intentional exposure, there is a risk of unintentional exposure through the use of certain medications, creams, drinking water, etc. (AREDS, 2002; Hedera et al., 2009; Dietrich et al., 2004).

Biometal supplementation has also been linked to altered behavior in rats and mice. Zn supplementation (via drinking water) has been linked to altered fear response and spatial memory in rats, and spatial memory deficits in Tg2576 and CRND8 mice (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). Additionally, three of the aforementioned studies found that the addition of Cu to the enhanced Zn water remediated the negative effects seen in the enhanced Zn group (Chrosniak et al. 2006; Railey et al., 2010; Railey et al., 2011). These findings lend support to the theory that excess Zn may be causing impairments due to an induced Cu deficiency. Large amounts of Zn intake relative to Cu can cause a deficit in Cu absorption; without an adequate amount of Cu to regulate physiological actions, consequences include oxidative stress, abnormal regulation of cholesterol homeostasis, and dysregulation of Fe transport and metabolism (Dharmarajan et al., 2012; Maret, & Sandstead, 2006). This relatively simplistic cause and effect scenario may not be

sufficient to explain the behavior. A direct mild Cu deficiency in an EOAD model improved performance on the MWM when compared to a Zn-enhanced group; however, this was not the case for novel object recognition (Howell, 2014). These data indicate that the effects of Zn and Cu may be dependent on different brain structures, and that the Zn effect is not entirely due to an induced Cu deficiency (2014).

In order to observe behavioral changes resultant from biometal dysregulation, we used the three chamber social behavior test and Morris water maze as behaviors of interest, with open field and odor habituation/dishabituation as control experiments. The social behavior task, specifically the third trial, may serve as a measure of declarative memory and recognition memory, which have both been identified as dysfunctional in AD patients. It may more accurately represent disruption of function in areas of frontal and temporal cortices, which are thought to be involved in social cognition; traits that tend to decline in AD patients (Amodio & Frith, 2006; Frith & Frith, 2001; Frisoni et al., 1999). Morris water maze was used as a measure of spatial learning and memory. Mice were tested at both 6 and 12 months of age to identify short and long term effects of dietary manipulations, as well as investigate the potentially protective effects of the apoE4 allele early in life. Transgenic (Tg) mice modeling late onset AD (CRND8/E4) have exhibited intermediate levels of circadian rhythm disruptions and levels of IL-1β (an inflammatory cytokine) and glial fibrillary acidic protein (GFAP) as compared to an early onset model (CRND8) and wildtype (Wt) controls (Graybeal et al., 2015). Some E4 bigenic mice (hAPP-Yac/apoE4-TR) have also displayed an increased performance level in the Morris water maze (MWM) (Moreau et al., 2013). In humans, young, healthy

apoE4 carriers have exhibited superior performance in tasks of attention, episodic memory and neural efficiency, recall, and have higher IQ's, (Rusted et al., 2013; Mondadori et al., 2007; Jochemsen, Muller, van der Graaf, & Geerlings, 2012; Yu, Lin, Chen, Hong, & Tsai, 2000). Older E4 carriers may also show a greater recruitment of brain areas during cognitive performance, perhaps to compensate for losses (Han, & Bondi, 2008). These data coincide with the idea of antagonistic pleiotropy in which a gene or allele may differentially impact function in various stages of life (Tuminello, & Han, 2011).

In summary, the above variables were explored in an attempt to better understand the connection between biometal dysregulation and LOAD. This study represented an important extension of the first study (mentioned above; Howell, 2014) that used an early onset mouse model, as these are the only studies to date that are investigating the effects of metal deficiency in AD directly through an altered diet. We have worked closely with Harlan Laboratory nutritionists to develop diets that can be directly compared with one another, due to their differing only in levels of one ingredient, i.e. Cu; this allows us to make more clear inferences based on metal effects alone. Further importance lies in the longitudinal nature of this study. Many others look at short-term effects of manipulations, whether it be dietary or otherwise, in Tg mice, but few investigate the short and long term effects of such changes. The longitudinal design is especially important here in order to investigate the potentially protective effects of the E4 allele.

CHAPTER TWO: MATERIALS AND METHODS

Subject Mice:

Male 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) were crossed with Female mice heterozygous for hApoE4 to obtain a mouse model of LOAD (hAPP/hE4). The J20 strain was chosen due to its availability, common background strain, and double hAPP mutation that produces diffuse amyloid plaques by age 5-7 months and behavioral deficits by 4-7 months. C57Bl/6J (B6) mice were used as Wt controls. All mice used for experimental purposes were on a B6 background. All breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME); experimental mice were bred at George Mason University.

Harem breeding was utilized; paired for 14 days. Tail snips were collected from the pups between postnatal days 11-21 for genotyping (Transnetyx; Cordova, TN). Pups were weaned at postnatal day 21-30 according to sex and genotype with 2-6 mice per cage. Single housing was avoided whenever possible. Each cage contained two igloos, one with a wheel attachment and one without, and a nylabone for enrichment. Experimental animals were handled 3-4 times per week for the duration of the study (beginning 2 weeks post-weaning). All 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/Water:

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 and laboratory tap water upon weaning and their group specific diets/water at 8 weeks of age. 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.

Zn and Cu levels in 7012 were used as a guideline for the new hard diets in attempts to keep the Zn levels as close to our standard diet as possible (7012= ~63ppm Zn, new diet= 40ppm Zn). The T.7012 diet contains non-nutritive substances, such as phytates, which affects the absorption of minerals; because of this, minerals in T.7012 are generally higher than what would be needed for purified diets, such as the ones created for this experiment (T. Herfel PhD., Harlan industries, personal communication, March 12, 2013). Both experimental diets were 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 and constant level of Zn.

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 13 months of age, after 11 months of dosing.

Groups were given food and water as follows:

Table 1: *Experimental groups according to genotype, food and water*

Wildtype	**Transgenic (hAPP/hE4)**
Lab water+ Copper control	Lab water+ Copper control
Zinc water+ Copper control	Zinc water+ Copper control
Lab water+ Copper deficient	Lab water+ Copper deficient

Behavioral Testing:

Experiment 1: Pro-Social Behavior

Social behavior was assessed at just over 6 and 12 months of age. Two subject mice were tested at a time in separate testing boxes. Testing boxes were (17" x 25" x 10"). Each box was divided into three compartments by two pieces of clear Plexiglas with removable doors attached (Clever Sys., Inc., Reston, VA). Each outside chamber contained a wire cup to house "stranger mice" for the duration of trials 2 and 3 (age/sex matched 129S6/SvEvTac (Taconic, Hudson, NY)). 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 wire cup. Animals were run in random order and counterbalanced. Boxes were cleaned with 70% ethanol in between subject mice. Testing consisted of three trials: habituation, sociability, and preference for social novelty.

Habituation: The subject mouse was confined to the center chamber until the doors were removed. Once the doors were removed, the subject mouse was free to explore all 3 chambers of the testing box for 10 minutes.

Sociability*:* After 10 minutes of habituation, the doors separating the chambers were closed, and the subject mouse was confined to the center compartment. A pre-determined "stranger" mouse was then placed inside of a wire cup in one of the side chambers. Once the "stranger" was in place, the doors were removed, and the subject mouse was free to explore all three chambers for 10 minutes.

Preference for Social Novelty: After 10 minutes of exploration, the doors separating the chamber were closed, and the subject mouse was confined to the center compartment. An additional "stranger" was then added to the empty cup in the second side chamber. Once the second "stranger" was in place, the doors were removed, and the subject mouse was free to explore all three chambers for 10 minutes.

Table 2. Groups of tested Animals: Social Behavior (6 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=20	n=17	n=25	n=62
Tg	n=23	n=19	n=23	n=65
Total	n=43	n=36	n=48	n=127

Table 3. Groups of tested Animals: Social Behavior (12 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=18	n=14	n=22	n=54
Tg	n=19	n=17	n=22	n=58
Total	n=37	n=31	n=44	n=112

Experiment 2: Morris Water Maze (MWM)

MWM was conducted three days following the completion of the social behavior task, when mice were approximately 6.5 and 12.5 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 tempera 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, 1x 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.

Latency to platform, platform crossings on probe trials, percent time spent in target quadrant on probe trials, and thigmotaxis were assessed. Thigmotaxis 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. Thigmotaxis 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.

***12-month testing**: Start locations and cues were randomly rotated. Due to limitations of the testing room, the platform remained in the same location at 6 and 12-months.

Table 4. Schedule of Morris Water Maze Days

Day	Number of Trials	Platform Location	Max. Trial Length	Inter-trial 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 5. Groups of tested Animals: MWM (6 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=19	n=20	n=22	n=61
Tg	n=23	n=19	n=23	n=65
Total	n=42	n=39	n=45	n=126

Table 6. Groups of tested Animals: MWM (12 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=18	n=16	n=19	n=53
Tg	n=23	n=15	n=22	n=60
Total	n=41	n=31	n=41	n=113

Experiment 3: Open Field (OF)

Open Field was conducted following the MWM task when mice were just over 6.5 and 12.5 months of age. Each animal received one, 10-minute trial in which they were free to explore an open field testing apparatus (blue plastic box, measuring approximately 20in X 20in X 20in; CleverSys, Inc.). TopScan analysis (CleverSys, Inc.) was used to calculate total distance traveled, as well as average velocity. Testing boxes were cleaned with 70% ethanol in between animals.

Table 7. Groups of tested Animals: Open Field (6 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=20	n=20	n=22	n=62
Tg	n=23	n=19	n=23	n=65
Total	n=43	n=39	n=45	n=127

Table 8. Groups of tested Animals: Open Field (12 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=15	n=11	n=15	n=41
Tg	n=13	n=11	n=18	n=42
Total	n=28	n=22	n=33	n=83

Experiment 4: Olfactory Habituation/Dishabituation(OHD)

Odor habituation/dishabituation testing was conducted after completion of open field when mice were approximately 7 and 13 months of age. Mice were placed in individual testing cages with wire lids. Consistent with Yang & Crawley (2009), the testing consisted of two phases: habituation and testing.

Habitutation phase: Mice were presented with a clean, dry, 6in. wooden cotton-tipped applicator through the water bottle hole in a metal cage top. This phase lasted for 30 minutes and occurred in a room separate from the testing room.

Testing phase: Mice were moved from the habituation room and taken to the testing room, where they were presented with 5 distinct odors (3 non-social and 2 social) on the cotton-tipped applicators. Each odor received 3x, 2 minute trials with a 1 minute inter-trial interval (a total of 15 trials). Applicators were replaced for every trial. All non-social odors were prepared fresh each day. Dirty bedding (age/sex matched mouse cages) was

used for the social odor presentations.

Table 9. Odor Presentations

Trial	Type	Odor
1-3	Non-social	Distilled water
4-6	Non-social	Almond extract (1:100 dilution)
7-9	Non-social	Banana extract (1:100 dilution)
10-12	Social	Dirty bedding swab (cage 1)
13-15	Social	Dirty bedding swab (cage 2)

Table 10. Groups of tested Animals: Odor (6 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=19	n=18	n=21	n=58
Tg	n=18	n=10	n=19	n=47
Total	n=37	n=28	n=40	n=105

Table 11. Groups of tested Animals: Odor (12 months)

	Cu Control + Lab Water	Cu Deficient + Lab Water	Cu Control + Zn Water	Total
Wt	n=12	n=12	n=12	n=36
Tg	n=12	n=12	n=12	n=36
Total	n=24	n=24	n=24	n=72

CHAPTER THREE: RESULTS

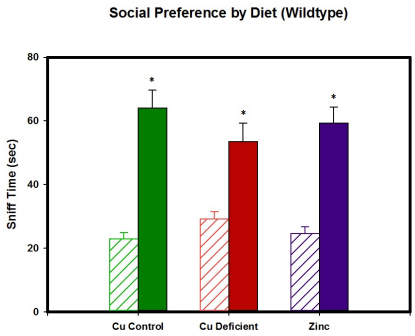
Experiment 1: Pro-Social Behavior

6 months:

Paired-samples t-tests were conducted to compare the amount of time spent sniffing the empty cup vs. the mouse cup for each genotype/diet condition (trial 2). Tests revealed that all mice display normal social behavior, spending significantly more time sniffing the "mouse cup" than the empty cup ($p<.001$ for all conditions) (Figure 1A and 1B). Paired samples t-tests were also conducted to compare the amount of time spent sniffing the "standard mouse" cup vs. the "novel mouse" cup during the preference for social novelty trial (trial 3). Statistics showed that, of the three wildtype diet conditions, only Cu controls spent significantly longer with the novel mouse, indicating a normal preference for novelty; $t(24)=1.607$, $p<.001$ (Figure 1C). Among the hAPP/hE4 diet conditions, Cu deficient and Zn mice prefer the "novel mouse" cup ($t(18)=2.459$, $p=.024$; $t(22)=3.221$, $p=.004$) (Figure 1D). Results for the trial 3 may be affected by a lack of odor sensitivity in some group conditions (Experiment 4: Olfactory Habituation/Dishabituation below). During the habituation trial, no significant differences were indicated for the duration of time spent in the left and right side chambers, $p>.05$ for all conditions (see Figure 13 in the appendix).

Figure 1.

A.



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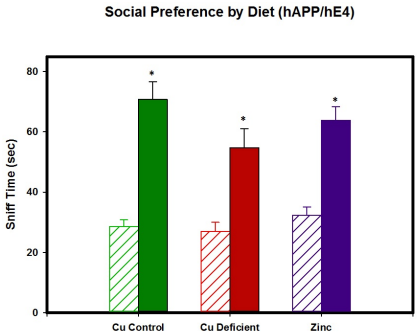

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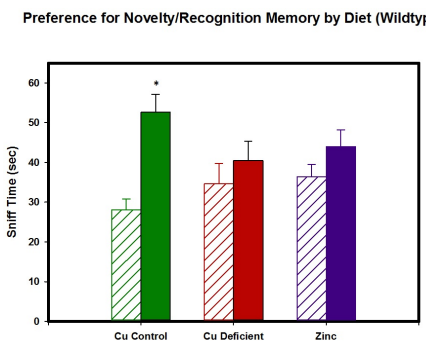

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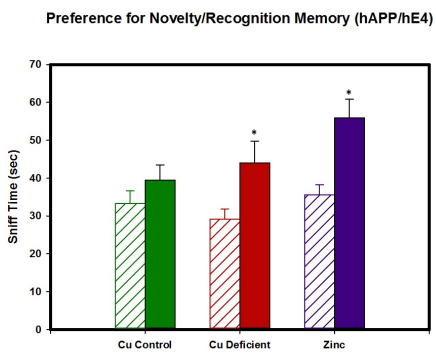


Figure 1. Social Behavior at 6 months.
All mice display normal social behavior, $p<.001$. Wt Cu control, Tg Zn and Tg Cu deficient mice show a normal preference for a novel stranger mouse during trial 3, $p<.05$.

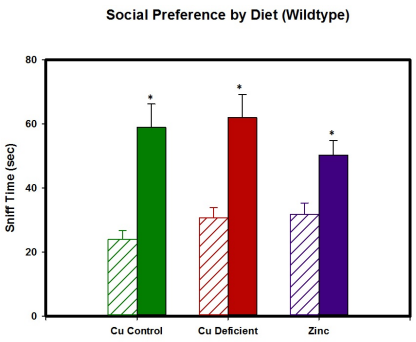
12 months:

Paired-samples t-tests comparing time spent sniffing the empty cup vs. the mouse cup revealed that all mice display normal social behavior, spending significantly more time sniffing the "mouse cup" than the empty cup ($p<.05$ for all conditions) (Figure 2A and 2B). No significant differences were seen between the time spent sniffing the "standard

mouse" cup vs. the "novel mouse" cup during trial 3 (preference for social novelty); p>.05 for all conditions (Figure 2C and 2D). During the habituation trial, no significant differences were indicated for the duration of time spent in the left and right side chambers, p>.05 for all conditions (see figure 13 in the appendix).

Figure 2.

A.



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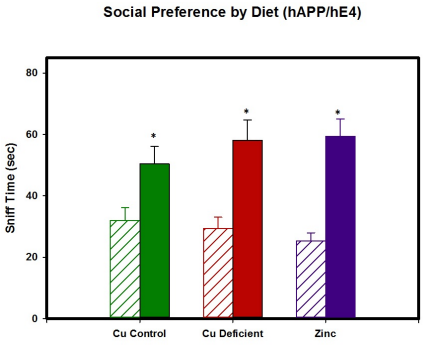

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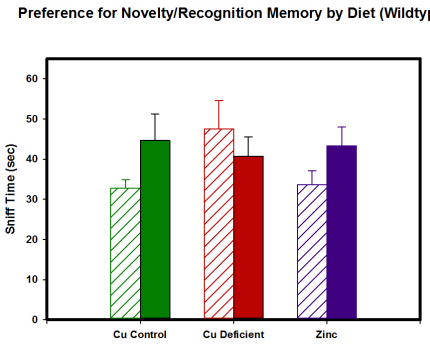

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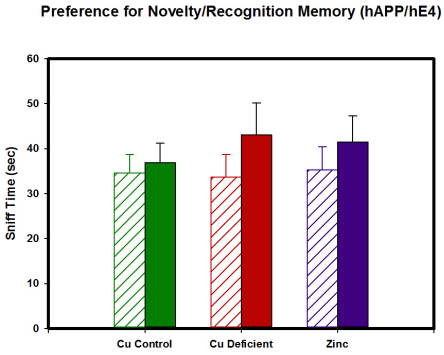


Figure 2. Social Behavior at 12 months.
All mice display normal social behavior, p<.05. No significant differences were seen during the preference for social novelty phase (trial 3), p>.05 for all conditions.

Experiment 2: Morris Water Maze (MWM)

6 months

A 2x3x6 (Genotype x Diet x Day) mixed ANOVA on average latency to the escape platform showed a significant effect of day ($F(4.460,535.150)= 45.775$, $p<.001$) and a significant between-subjects effect of genoptype ($F(1,120)=113.260$, $p<.001$), with wildtype mice showing significantly faster escape latencies than hAPP/hE4 mice (Figure 3). A 2x3x4 (Genotype x Diet x Day) mixed ANOVA on platform crossings (probe trials) showed a significant effect of day ($F(3,363)= 14.186$, $p<.001$) and a significant between-subjects effect of genotype ($F(1,121)= 59.283$, $p<.001$), with wildtype mice showing significantly more platform crossings across days than hAPP/E4 mice (Figure 4). A 2x3x4 (Genotype x Diet x Day) mixed ANOVA on percent time spent in the target quadrant (probe trials) showed a significant effect of day ($F(2.584,312.622)= 5.646$, $p<.01$) and a significant between-subjects effect of genotype ($F(1,121)= 4.073$, $p<.05$), with wildtype mice spending a greater percentage of time in the target quadrant than hAPP/E4 mice (Figure 5). A 2x3x7 mixed ANOVA on thigmotaxis showed a significant effect of day ($F(4.599,556.465)= 88.165$, $p<.001$) and a significant between-subjects effect of genotype ($F(1,121)= 114.167$, $p<.001$) with hAPP/hE4 mice showing more anxiety-like behavior than wildtypes (Figure 6). This measure may also indicate a lack of efficient search strategy for the transgenic mice. Overall, these data indicate that, at 6 months, all mice learn the task as the trials progress, but the wildtype mice learn the task faster than do the hAPP/E4 mice. Results could be affected by increased thigmotaxis in the transgenic conditions. No significant effects of diet were seen at the 6-month time

point (see Figures 14-18 in the appendix).

Figure 3.

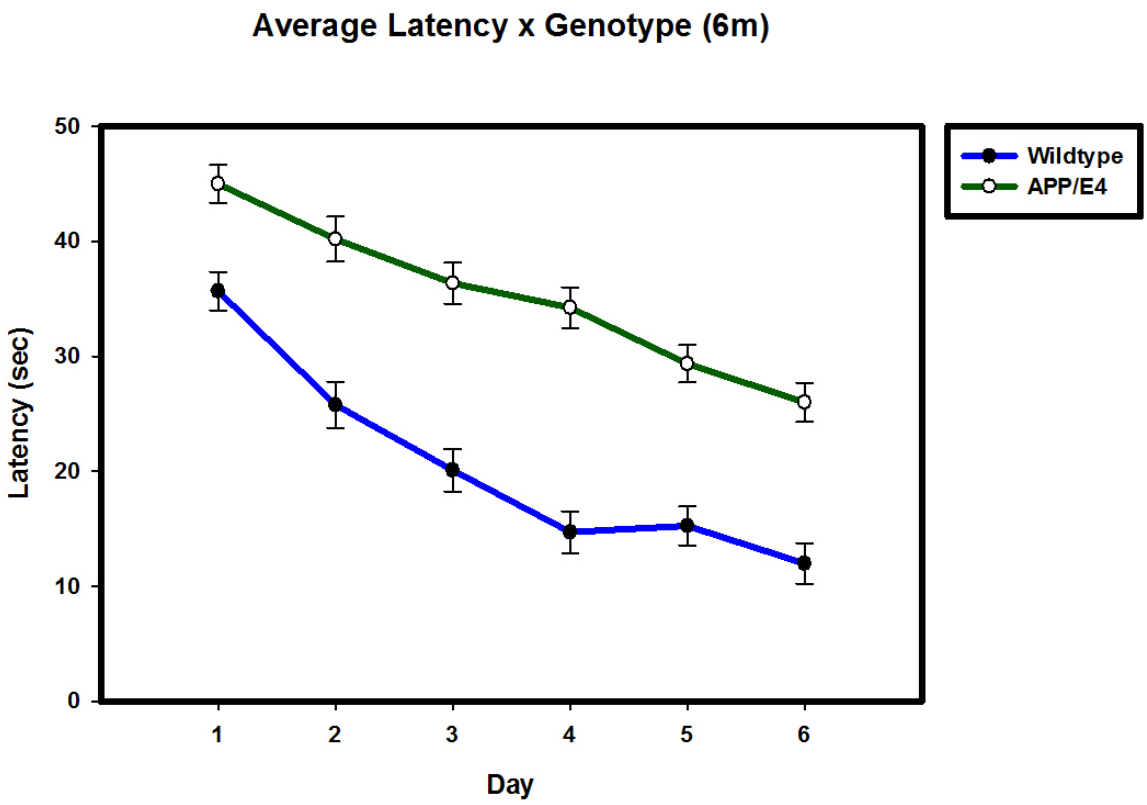


Figure 3. Average Latency to the Escape Platform at 6 months.
Wt mice exhibit significantly faster latencies to the escape platform than Tg mice, p<.001.

Figure 4.

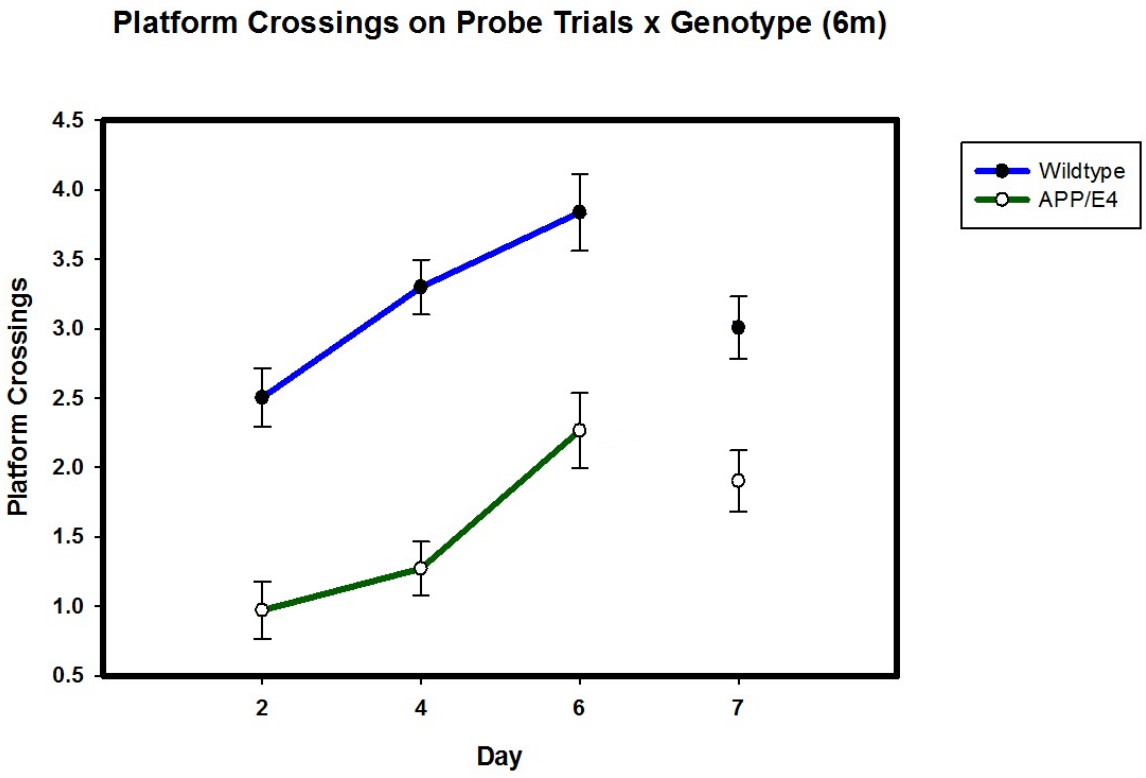


Figure 4. Average Platform Crossings at 6 months.
Wt mice exhibit significantly more platform crossings on probe trials than Tg mice, $p<.001$.

Figure 5.

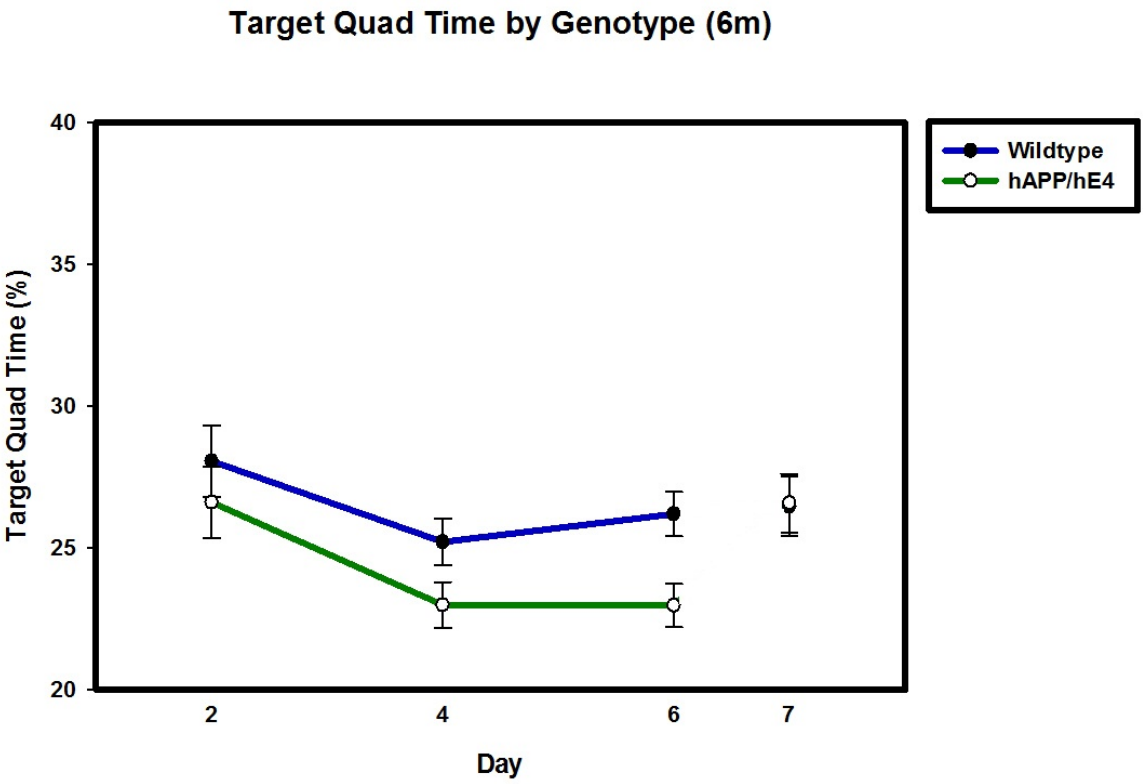


Figure 5. Average Time Spent in Correct Quadrant at 6 months.
Wt mice spend significantly more time in the correct quadrant during probe trials than Tg mice, $p<.05$.

Figure 6.

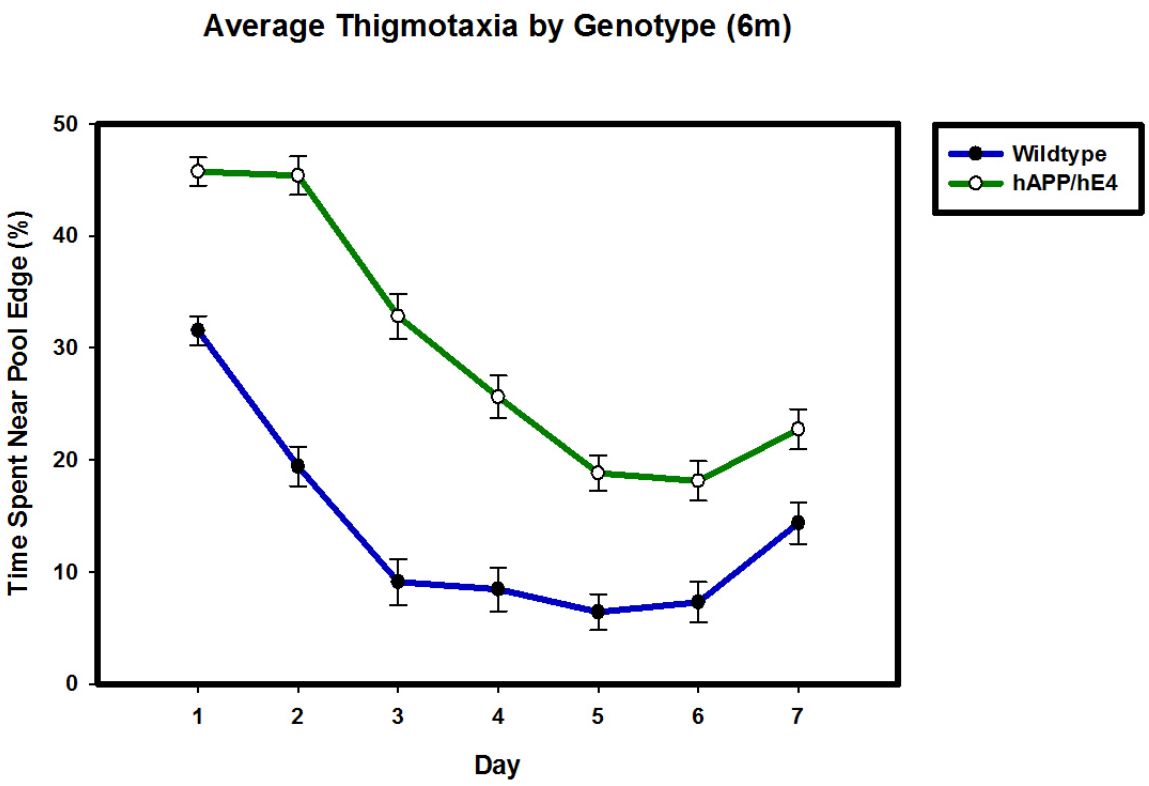


Figure 6. Average Thigmotaxis at 6 months.
Tg mice spend significantly more time around the outermost 10% of the pool than Wt mice, $p<.001$.

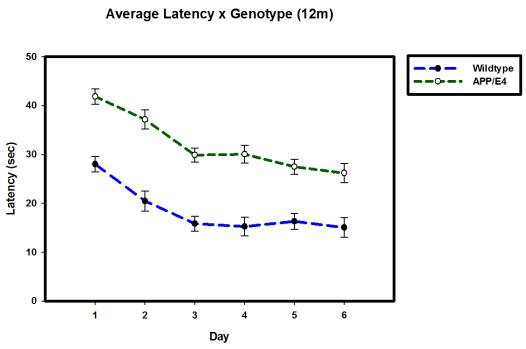
12 months:

Mixed ANOVAs on average latency, platform crossings on probe trials, and percent time spent in the target quadrant revealed a significant effect of day and a significant between-subjects effect of genotype, with wildtype mice outperforming hAPP/hE4 mice on all measures ($p<.001$) (Figure 7A-C). Data also revealed a significant effect of day (decreasing as days progress) and a significant effect of genotype for the thigmotaxis

measure, with hAPP/hE4 mice spending significantly more time around the outermost 10% of the pool ($p<.001$) (Figure 7D). No significant effects of diet were indicated (see Figures 14-18 in the appendix). Comparison of six and 12-month data indicate a potential practice effect, due to 12-month Tg mice showing significant improvement on latency with age ($F(1,123)=4.482$, $p<.05$) (Figure 7A). Wildtype mice also show a trend towards improvement on latency ($F(1,113)=3.362$, $p=.069$) and spend a significantly higher proportion of time in the target quadrant on probe trials ($F(1,113)=10.421$, $p<.01$) as they age (Figure 7A-B). Both genotypes display a significant decrease in thigmotaxis from 6 to 12 months, $p<.001$ (Figure 7C).

Figure 7.

A.



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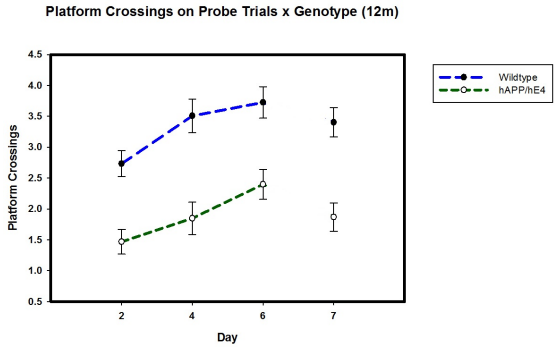

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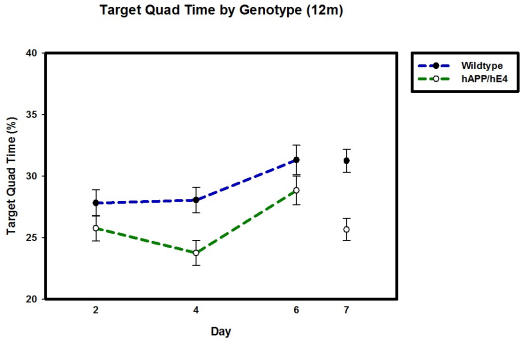

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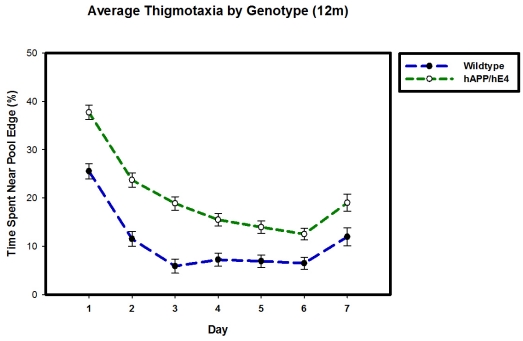
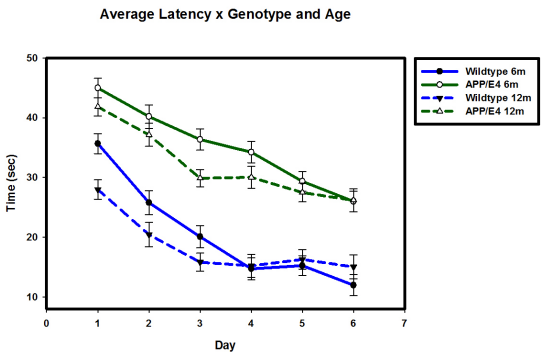


Figure 7A-C. Water Maze Measures at 12 months.
(A-C) Wildtype mice outperform hAPP/hE4 mice on average latency, platform crossings on probe trials, and percent time spent in the target quadrant, p<.001. (D) Tg mice spend significantly more time around the outermost 10% of the pool than Wt mice, p<.001.

Figure 8.

A.



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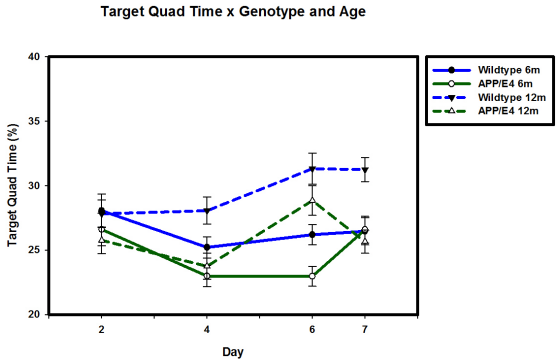

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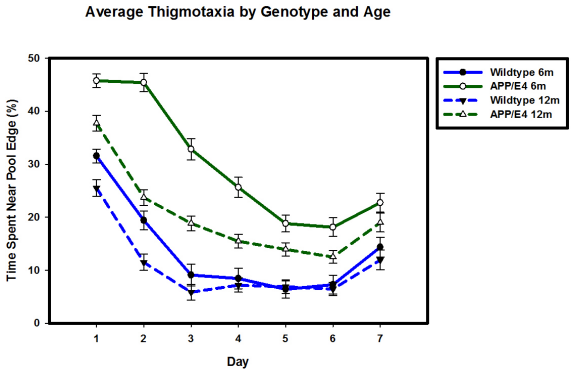

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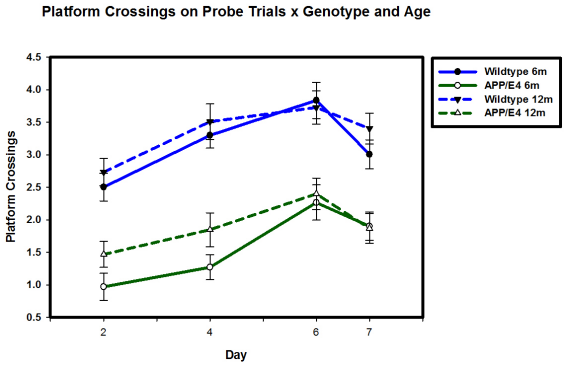


Figure 8. 6 and 12 Month Age Comparisons.
(A) Tg mice show significant improvement on latency with age, $p<.05$. (B) Wt mice show significant improvement on time spent in the target quadrant on probe trials, $p<.01$. (C) Both genotypes show significantly reduced thigmotaxis at 12 months, $p<.001$. (D) No significant improvement was seen on platform crossings.

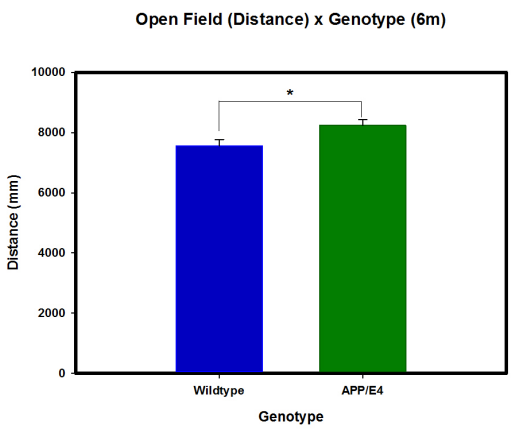
Experiment 3: Open Field

6 months

Six month open field data showed a significant between-subjects effect of genotype for distance traveled and average velocity, with hAPP/hE4 mice displaying more hyperactivity than wildtype mice ($F(5,121)=5.477$, $p<.05$; $F(5,121)=5.273$, $p<.05$) (Figure 9A-B). No significant effects of diet were seen at the 6-month time point.

Figure 9.

A.



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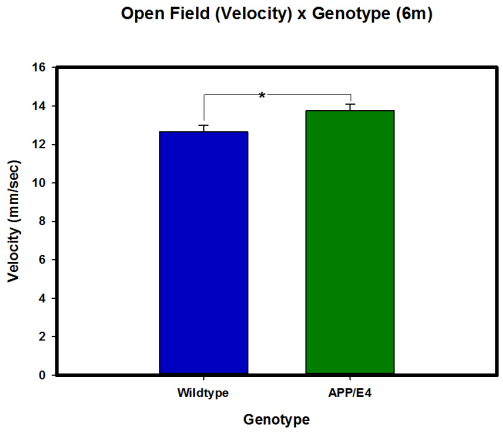


Figure 9. Average Distance Traveled and Velocity at 6 months.
Tg mice show increased hyperactivity when compared to Wt's, traveling significantly longer distances and at faster speeds, $p<.05$.

12 months:

At 12 months, no significant differences were seen between genotype or diet conditions for distance traveled and average velocity (p>.05) (Figure 10A-B).

Figure 10.

A. B.

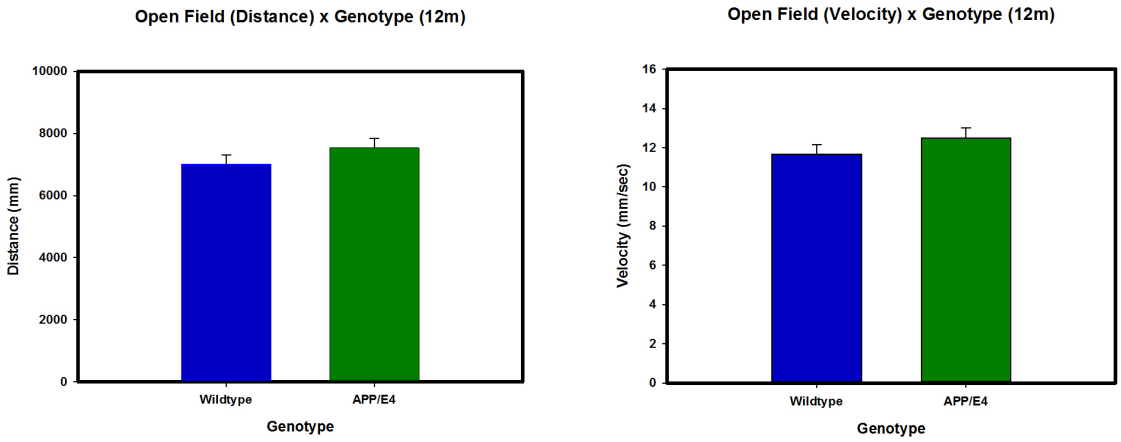


Figure 10. Average Distance Traveled and Velocity at 12 months.
No significant differences in average distance traveled or velocity were seen at 12 months, p>.05.

Experiment 4: Olfactory Habituation/Dishabituation (OHD)

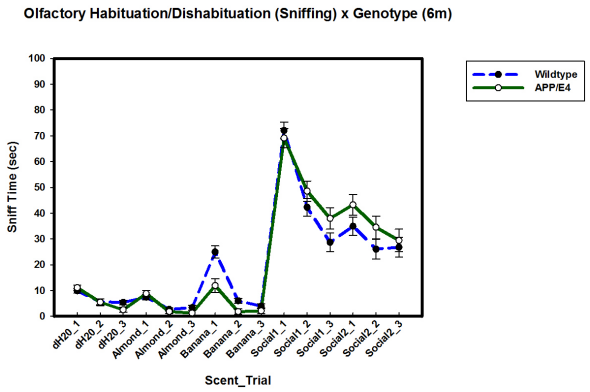
6 months:

At 6 months, 2x3x3 (Genotype x Diet x Trial) mixed ANOVAs on sniff duration showed that all animals exhibit normal habituation and dishabituation to non-social odors (p<.01) (Figure 11). Mixed ANOVAs on sniff duration for social odors revealed a significant interaction between genotype and diet for the second social odor (F(2,99)=3.982, p<.05). All mice display normal habituation to the first social odor

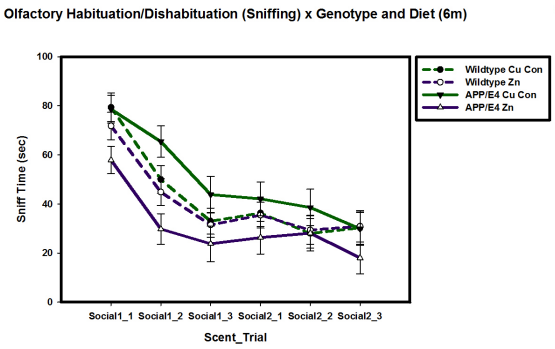
(p<.001); however Wt and Tg Cu control and Zn mice do not dishabituate to the second social odor, nor do they habituate to that odor upon repeated presentations (p>.05) (Figures 11B-C).

Figure 11.

A.



B.



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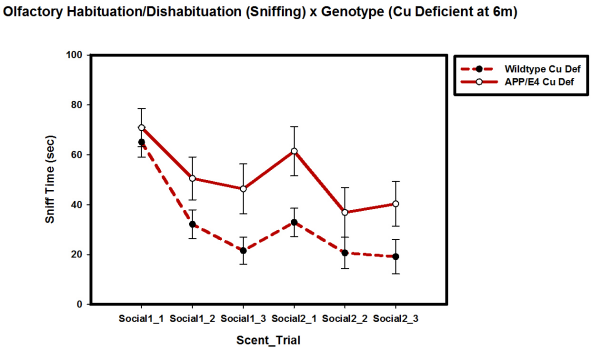


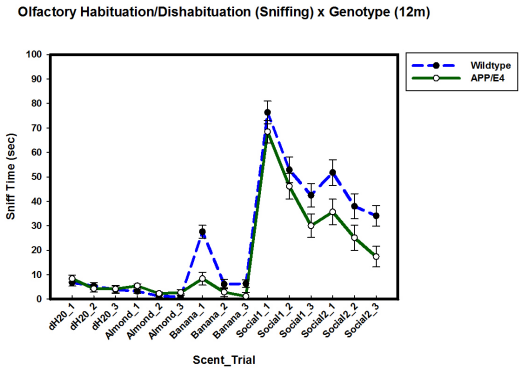
Figure 11. Sniffing Behavior at 6 months.
(A) All mice exhibit normal habituation/dishabituation to non-social odors, p<.001. (B) Wt and Tg Cu control and Zn mice do not dishabituate to the second social odor, nor do they habituate to that odor upon repeated presentations, p>.05. (C) Wt and Tg Cu Deficient mice exhibit normal habituation/dishabituation to the social odors, p<.05.

12 months:

At the 12-month time point, mixed ANOVAs on sniff duration, showed that all mice display normal habituation and dishabituation to non-social odors ($p<.05$) (Figure 12). Mixed ANOVAs on sniff duration for social odors, revealed a significant between-subjects effect of genotype for the second social odor ($F(1,66)=8.517$, $p<.001$), with Wt mice showing significantly increased sniffing behavior. Additionally, there was a significant effect of diet, where in the case of both social odors, Wt and Tg Cu control mice sniff the swabs significantly more than Cu deficient and Zn mice ($p<.01$). Neither Cu deficient nor Zn mice (both genotypes) dishabituate to the second social odor ($p>.05$) (Figure 12B-C).

Figure 12.

A.



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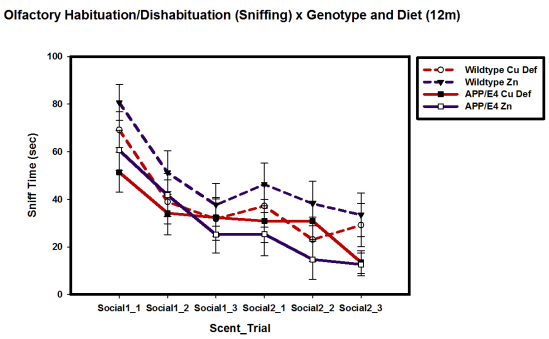

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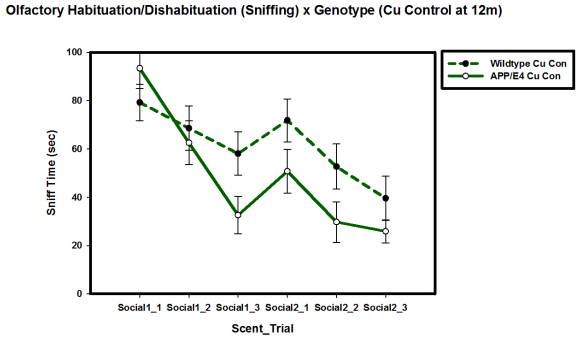


Figure 12. Sniffing Behavior at 12 months.
(A) All mice exhibit normal habituation/dishabituation to non-social odors, p<.001. For the social odors, Wt mice show significantly more sniffing behavior than Tg's, p<.001. Wt and Tg Cu control mice sniff the social swabs significantly more than Cu deficient and Zn mice, p<.01. (B) Wt and Tg Cu deficient and Zn mice do not dishabituate to the second social odor, p>.05. (C) Wt and Tg Cu control mice exhibit normal habituation/dishabituation to the social odors, p<.05.

CHAPTER FOUR: DISCUSSION

The primary purpose of this study was to identify both short and long term behavioral alterations, resultant from manipulations of Zn and Cu, in a mouse model of LOAD. A limitation of many AD studies is the reliance on an early onset model, which ignores a vast majority of AD cases. Additionally, few studies have looked at behavioral changes longitudinally, testing mice in early and late stages of the disease.

Results from the 3-chamber test suggest that, in this model, there are no impairments in sociability at either time point, but preference for social novelty may be affected by experimental diet conditions in early stages of the disease. Tg Cu deficient and Zn mice prefer the novel mouse at 6 months, as does the Wt Cu control condition. This suggests that a higher Zn to Cu ratio may improve social behavior in hAPP/hE4 mice. AD has been associated with Zn deficiency and a decrease in odor sensitivity, so an excess amount of Zn may uniquely improve Tg mouse behavior in a task that relies on distinguishing odors; Zn would not be expected to improve performance for the Wt's, as they are not considered Zn deficient. OHD data contradicts these results, indicating that Tg Zn mice do not exhibit normal sniffing behavior; however, this could be affected by boredom during the olfactory test itself. No significant differences were seen in the preference for social novelty phase at 12-months, which may be the result of a significant disruption in odor sensitivity at an older age; such an age-dependent decline has been

documented in AD mouse models, as well as wildtypes (Wesson et al., 2011; Mobley, Rodriguez-Gil, Imamura, & Greer, 2014). Specifically, perinatal rodents exhibit a 90% reduction in olfactory epithelium between 3 and 16 months (Mobley et al., 2015). This warrants further investigation into dysfunction of the olfactory system in this model.

Analysis of spatial learning and memory via MWM showed a significant difference between Wt and Tg mice at both ages. While the genotypes remain significantly different at 12-months, latency results indicate that Tg mice significantly improve with age, which was unexpected. Wildtype mice also showed a trend towards faster latencies and a significant improvement in time spent in the target quadrant during probe trials at 12-months. Despite the movement of the cues for the 12-month testing, practice effects may have had serious implications for identification of deficits in later stages of the disease. The presence of practice effects is highlighted by the significant decrease in thigmotaxis by both genotypes. MWM did not indicate any group specific differences in diet, i.e. metals. These results are inconsistent with a previous study using the same diets that indicated a mildly protective effect of Cu deficiency in an early-onset model (Howell, 2014) (see Figure 19 in the appendix). Additionally, these results contradict another study that showed a significant impairment in spatial memory (via Barnes maze) with Zn supplementation in a late-onset model (Flinn, Bozzelli, Adlard, & Railey, 2014).

When looking at behavioral results from other studies in the field, it is apparent that the issue is complex, and results are, as of yet, unclear. In regards to Cu, a precise index for too much or too little Cu is undefined; however, there are a limited number of studies that investigate the importance of its regulation on behavior. A study by Bolognin et al.,

(2012) utilized behavioral and biochemical assays to examine the role of dietary Cu deficiency on aged CD1 mice (diets were with and without 6mg cupric carbonate per kilo); no significant differences were indicated between the Cu deficient and control groups in regards to motor, sensory, emotional or cognitive behaviors. Additionally, Harris et al. (2014) utilized an indirect measure of Cu deficiency (adding excess Zn to the drinking water) in aged (at least 13 months) Tg2576 mice modeling AD. Their results showed no behavioral improvement in the Cu deficient group, as they hypothesized that there would be; this is despite a reduction in Aβ burden (2014). As for the issue of excess Cu, cholesterol fed rabbits modeling AD show impairments in hippocampal dependent conditioning tasks, as well as an increased number of Aβ plaques, after 8 weeks on excess Cu given in drinking water (Sparks, & Schreurs, 2003). On the contrary, adult male Wistar rats receiving injections of CuSO4 did not show deficits in the Morris water maze (MWM) despite having decreased long-term potentiation (LTP) and increased levels of brain Cu in the hippocampus and visual cortices (Leiva, Palestini, Infante, Goldschmidt, & Motles, 2009). Experimental manipulation of Zn levels has proven to be equally multifaceted. As previously mentioned, excess Zn has been linked to altered fear response and spatial memory in rats, and spatial memory deficits in mice with mutated hAPP; research has also shown impairments associated with Zn deficiencies. Zn chelators targeted to hippocampal CA3 mossy fibers disrupted the acquisition and consolidation of contextual fear conditioning (Daumas, Halley, & Lassalle, 2004). Moreover, animal studies on Zn deficiency during development or early adolescence show an increase in emotionality and susceptibility to stress, as well as impairment in

memory and capacity to learn (Bhatnagar, & Taneja, 2001; Halas, Hunt, & Eberhardt, 1986; Halas, Eberhardt, Diers, & Sandstead, 1983).

With such variable results, there are some points to consider; for example, the base diet being manipulated. The previously mentioned study by Flinn et al. (2014) used the T.7012 diet, which differs from the ones used for this study [and the early-onset study] (Howell, 2014). An additional consideration is the difference in strains of the animals used. This study [and Howell, 2014] used the J20 model from The Jackson Laboratory (C57 background strain); however, there are two notable differences that may have affected the results. The EOAD model did not contain the hE4 allele and the C57 control mice used for that study were littermates of the hAPP mice, while the LOAD study used separately bred controls. Comparison of latencies for all three models indicates a significantly faster rate of learning for the LOAD model wildtypes (see Figure 20 in the appendix). In regards to the other LOAD study mentioned, Flinn et al. (2014) used the CRND8 mouse, which uses the hybrid C3H/He-C57BL/6 background strain that the J20 does not.

Overall, this study indicates the importance of considering the mouse model, as well as the standard diet, when attempting to manipulate a system as delicately balanced as metal levels in the body/brain. Many of the previous studies indicating Zn deficits used the standard rodent diet T.7012 (Harlan Industries), which differed in metal levels, non-nutritive substances, and the presence of 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 high enough 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. Further, isoflavones found in soy can bind to estrogen receptors and possibly effect behavior. Related specifically to Zn and Cu, soy has been shown to block Zn uptake, thereby causing a Zn deficiency and a concomitant excess of Cu. 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. Analysis of the diets will be conducted in order to identify dietary interactions that may have contributed to the differences seen between the EOAD and LOAD model studies that used the newly created diets, and the previous studies that used the T.7012 diet.

In summary, when combined with results from others in the field, it is clear that manipulating metal levels in the body/brain via hard diets is more difficult than it may seem, especially when looking at mouse models of disease. The presence or absence of significant results may depend heavily on the type of diet used and the particular rodent

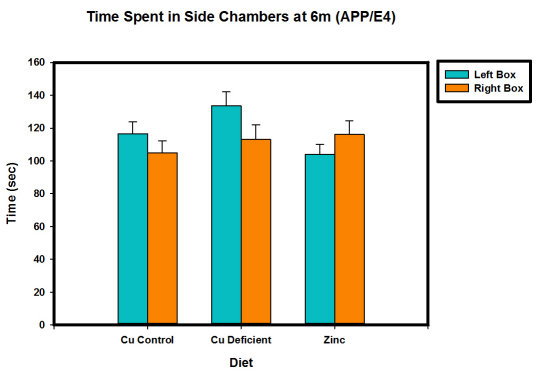
model in question. Adjusting the levels of ingredients (metals) in a rodent diet is complex; it is impossible to change one ingredient without affecting the levels and absorption of the others. Due to the concern of keeping rodents diets safe for consumption, the question remains, how translatable are the results? The average person does not take into consideration how changing one aspect of their diet can affect how their body reacts to the aspects that they haven't changed. So, while it may be more important than previously thought to control for the type of rodent 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), these studies should be interpreted carefully in relation to real-world disease scenarios.

APPENDIX

Experiment 1: Pro-Social Behavior Supplemental Figures:

Figure 13.

A.



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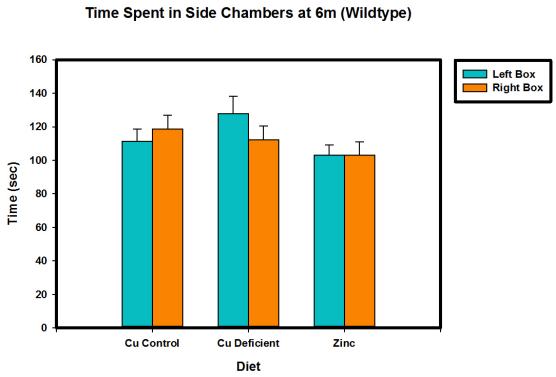
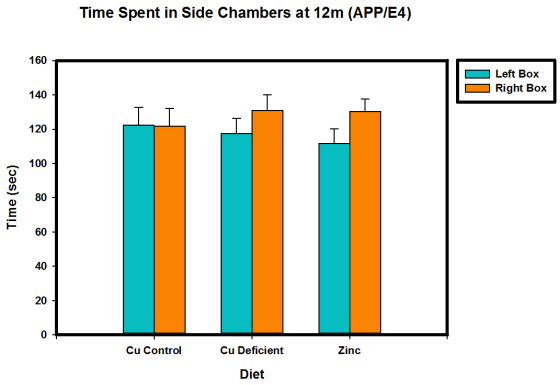


C.



D.

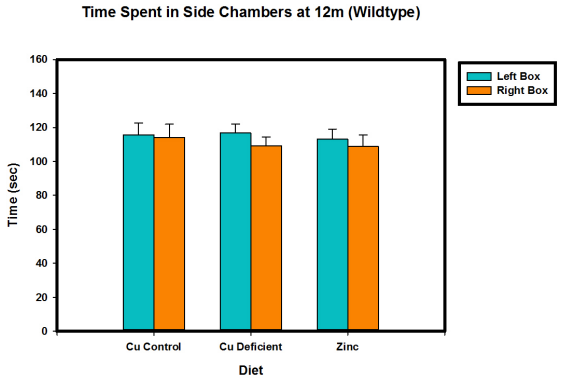


Figure 13. Time Spent in Side Chambers During Habituation.
During the habituation trial, no significant differences were indicated for the amount of time spent in each of the side chambers, $p>.05$.

Experiment 2: Morris Water Maze Supplemental Figures:

Figure 14.

A. B.

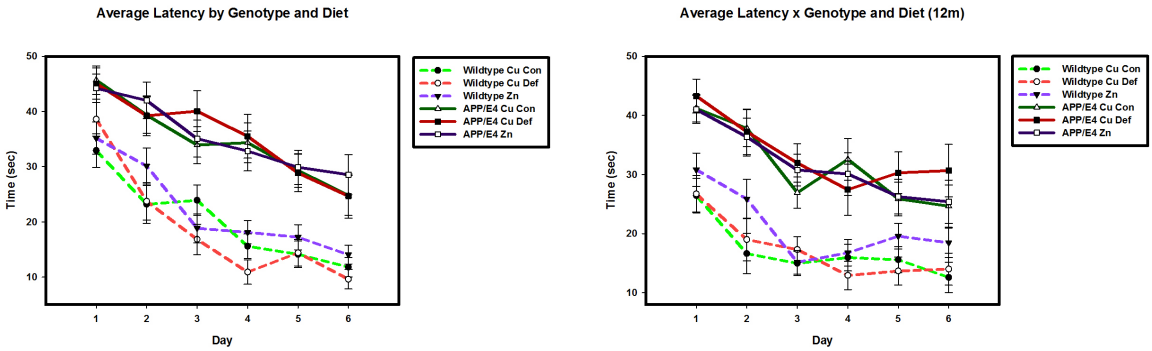


Figure 14. Latency by Genotype and Diet.
No significant differences in diet were indicated at 6 or 12 months, p>.05.

Figure 15.

A. B.

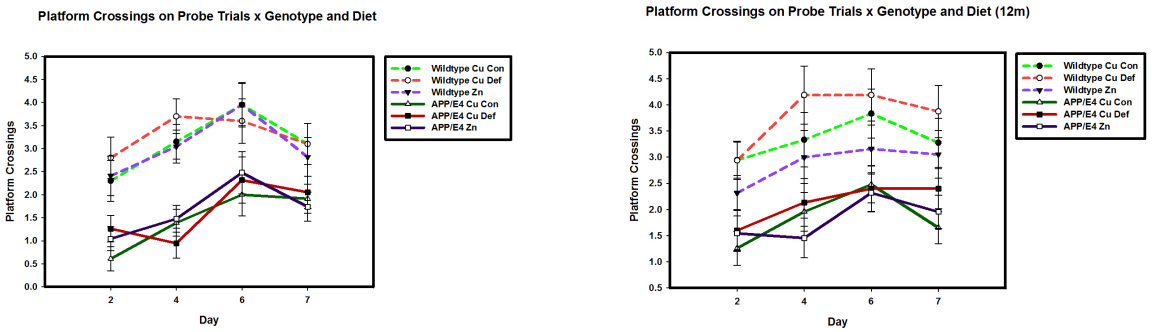


Figure 15. Platform Crossings by Genotype and Diet.
No significant differences in diet were indicated at 6 or 12 months, p>.05.

Figure 16.

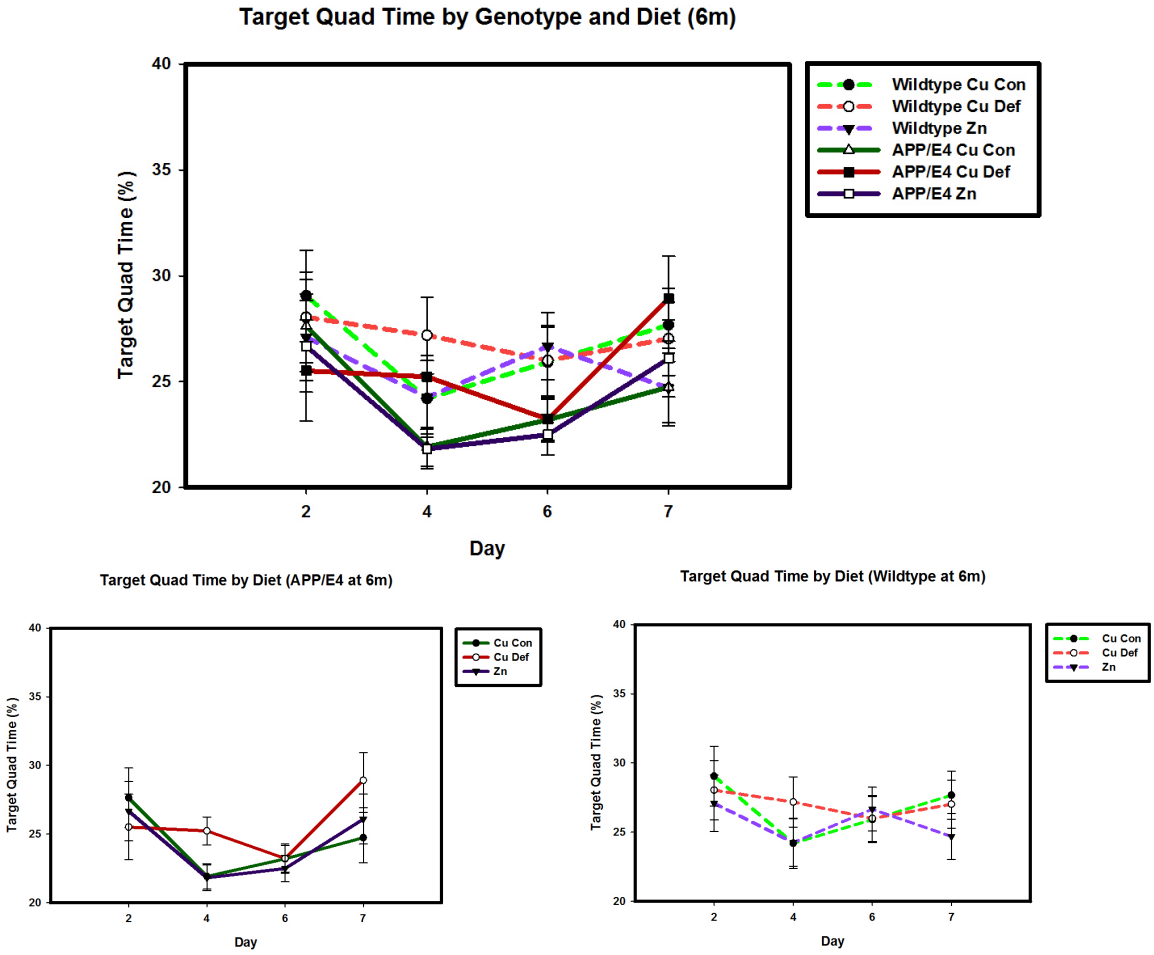


Figure 16. Target Quadrant Time by Genotype and Diet (6m).
No significant differences in diet were indicated, p>.05.

Figure 17.

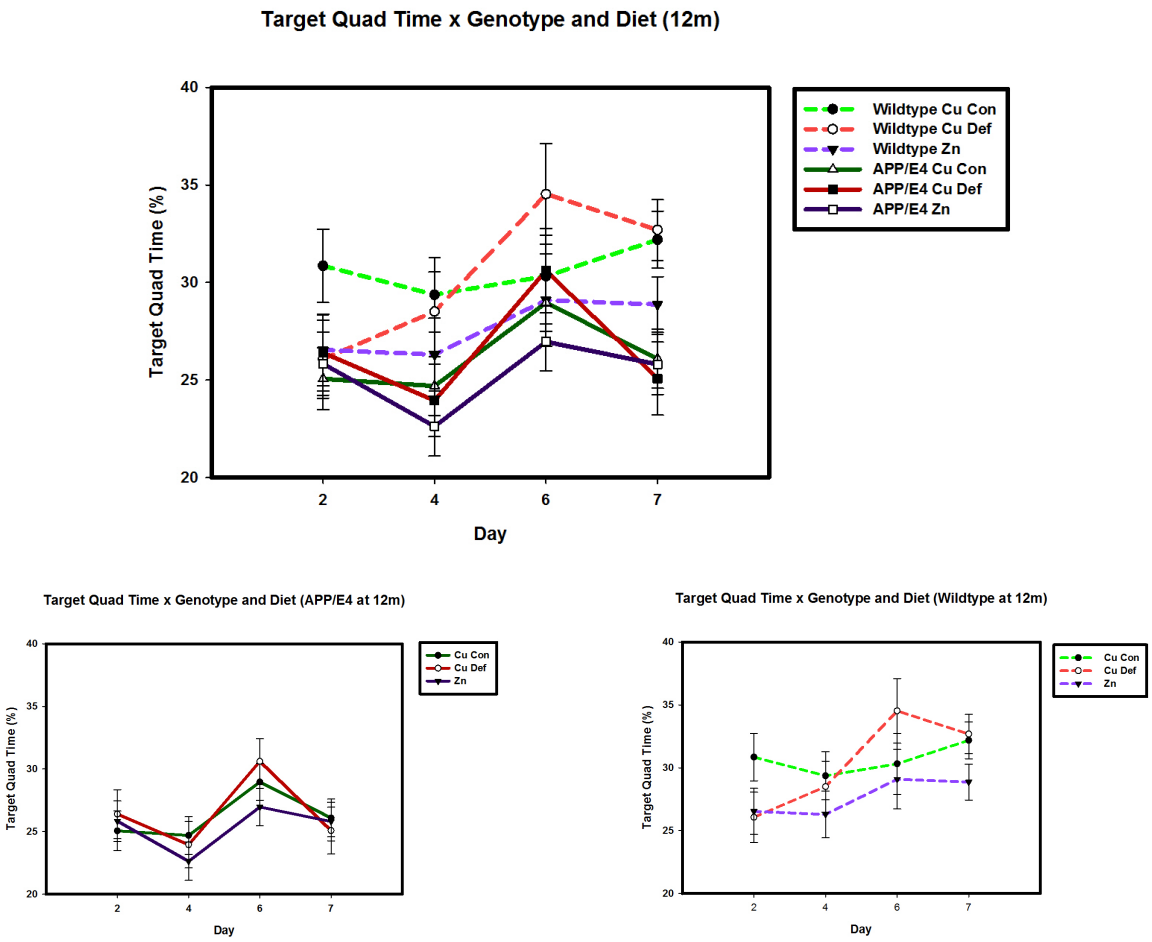


Figure 17. Target Quadrant Time by Genotype and Diet (12m).
No significant differences in diet were indicated, p>.05.

Figure 18.

A. B.

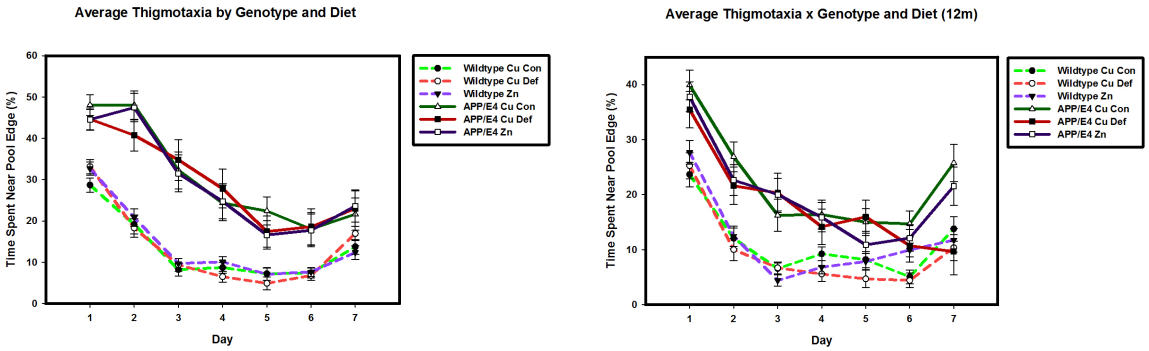


Figure 18. Average Thigmotaxis by Genotype and Diet.
No significant differences in diet were indicated at 6 or 12 months, p>.05.

Figure 19.

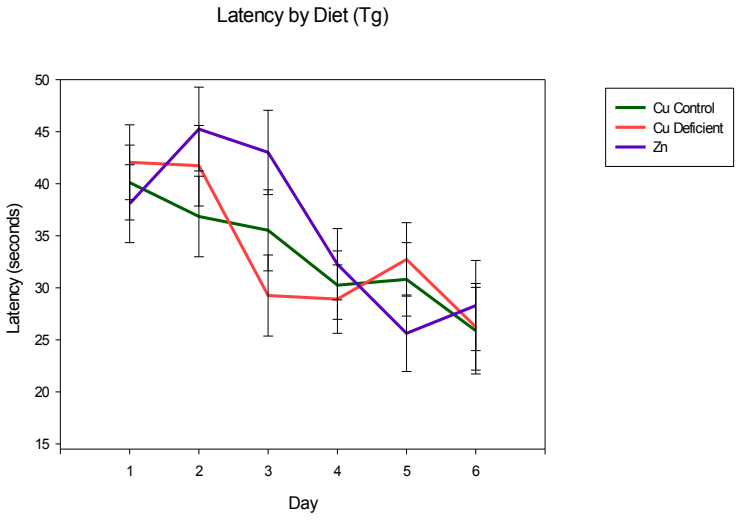


Figure 19. Average Latency by Genotype and Diet (EOAD Model).
Days 2-5 showed a significant interaction between day and diet (p<.05), driven by an interaction in the Tg mice (p<.05). Pairwise comparisons showed a marginally significant difference between the Cu deficient and Zn-enhanced groups on day 3, indicating a delay in learning for the Zn-enhanced mice (p=.059).

Figure 20.

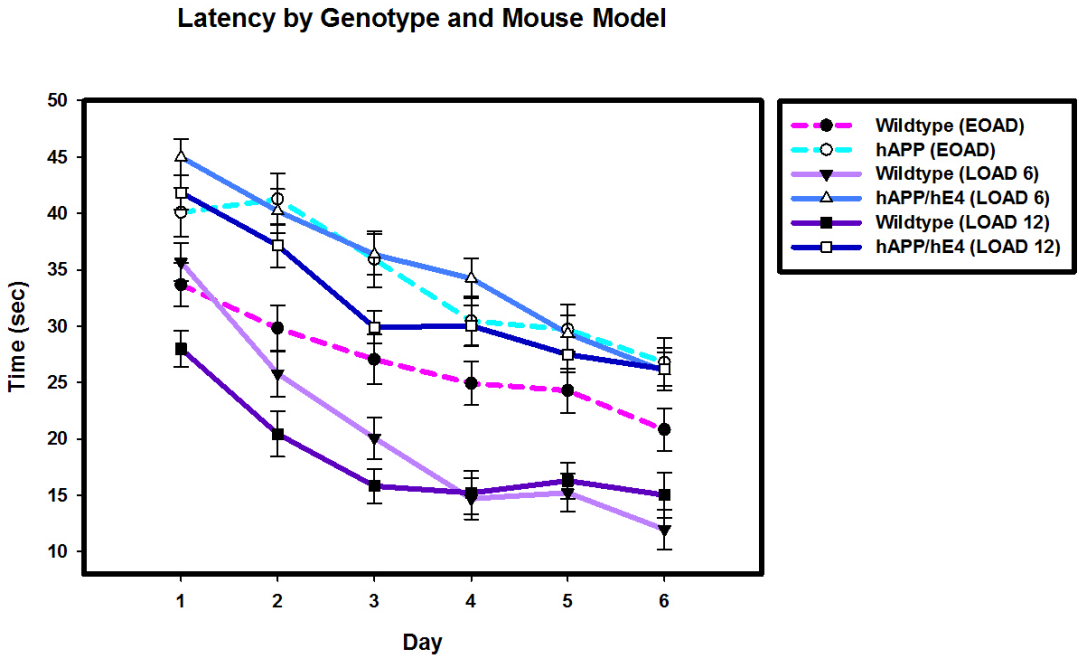


Figure 20. Average Latency by Mouse Model.
Comparison of data across models indicates a significant difference between the Wt mice used for the EOAD and LOAD models.

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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. 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 and graduated with her Master of Art's degree in Cognitive and Behavioral Neuroscience, at George Mason University in 2014. This dissertation serves as completion of her Doctor of Philosophy degree, concentration in Cognitive and Behavioral Neuroscience, at George Mason University, Fairfax, VA.