AN INVESTIGATION INTO THE ROLE OF IRON, COPPER, AND GENDER IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE.

by

Caitlin M. Groeber
A Dissertation
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Doctor of Philosophy
Psychology

Committee:

_____ Director

_____

_____

_____ Department Chairperson

_____ Program Director

_____ Dean, College of Humanities and Social Sciences

Date: _____ Spring Semester 2013
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Caitlin M. Groeber
Master of Arts
George Mason University, 2009

Director: Jane M. Flinn, Associate Professor
Department of Psychology

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DEDICATION

This dissertation is dedicated to my family for the unconditional love and support throughout the entire process of my graduate career. To my husband Matthew Travis for supporting me and standing by my side through all the initials: B.S., M.A., Ph.D., Dr. and Mrs. To my mother Ginger Groeber, my father Vernon Groeber, and my sister Emily Coates who give me the strength to pursue my life passions and are always there to provide words of love and encouragement.

The product of this research is dedicated to every older adult who still fights for their good health and cognition in old age and to be a productive member of society. Specifically, this goes to the two role models of vitality involved in my life: Virginia Coleman, born June 27, 1919, and Dorothy Lavoie, born April 3, 1920.

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Thank you to Ebube Utomi for animal care and coordination of colony maintenance. Without your help it would not have been possible to maintain the colony and my sanity.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS OR SYMBOLS

Alzheimer's disease ..... AD
Amyloid beta ..... Aβ
Amyloid precursor protein ..... APP
Copper ..... Cu
Iron ..... Fe
Iron plus copper ..... Fe+Cu
Lab water ..... LW
Morris water maze ..... MWM
Novel object recognition ..... NOR
Transgenic ..... Tg
Wild-type ..... Wt

ABSTRACT

AN INVESTIGATION INTO THE ROLE OF IRON, COPPER, AND GENDER IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE.

Caitlin M. Groeber, Ph.D.

George Mason University, 2013

Dissertation Director: Jane M. Flinn

Age-related diseases are more prevalent due to increasing longevity, with this comes increasing dietary supplementation to maintain a healthy lifestyle. Alzheimer's disease (AD) is the primary cause of dementia and the sixth leading cause of death in the United States. Previous studies of transgenic AD mouse models have found that long-term dietary supplementation of zinc (Zn) impairs spatial memory, while copper (Cu) shows remediation of this impairment. Iron (Fe) shows a similar impairment in these tasks, but the potential effects of remediation by Cu have not been studied. In humans, being female is a risk factor for cognitive impairment in AD, therefore it is important to examine gender differences in AD. To examine the effect and interaction of Fe and Cu on cognitive function, male and female transgenic (Tg)CRND8 and wild-type (Wt) mice were administered enhanced drinking water for 5 months: Fe (10ppm $FeNO_3$), Fe+Cu (10ppm $FeNO_3$ + 0.2ppm $CuNO_3$), Cu (0.2ppm $CuNO_3$), and LW. Assessments

included the novel object recognition task (NOR; discrimination task) and the Morris water maze (MWM; spatial memory). Histological analysis was conducted on the brain to examine the plaque load, metal content, ceruloplasmin levels, and amyloid levels in these mice. In NOR, Tg Fe+Cu-treated animals showed decreased object recognition compared to Tg LW animals, while Wt Fe+Cu-treated animals performed well. In MWM, only the Fe+Cu-treated animals did not have a decrease in latency and were significantly different from the LW, who were the fastest. Even in normal animals, high levels of Fe+Cu showed negative effects. CRND8 animals were impaired on all assessed measures of learning and memory (NOR and MWM) compared to Wt animals. Females and males perform differently in behavioral tasks dependent upon the type of task. The level of NOR differed between male and female Wt mice, but not Tg. This indicates that female Wt mice are better at this task than male Wt mice; however, once these animals develop signs of AD the difference between the sexes disappears. In contrast, in MWM, males swam faster to the platform than females, and spent less time around the edge of the pool. This was the most pronounced in Tg males compared to Tg females. Sex differences were seen across the varying behavioral tasks of learning and memory, emphasizing the importance of studying both sexes. Plaque load analysis indicated that Fe+Cu-treated and LW Tg animals had the highest plaque burden, while Cu-treated animals had the lowest plaque burden. Females had more plaque area than males, with female Fe+Cu animals showing the highest plaque load of all animals. This measurement of plaque burden may be not be a predictive measure of NOR, but it may be predictive of MWM and it may be due to location of plaque accumulation. The reason may be due to

the brain regions required for the completion of the task; MWM depends on the hippocampus, while the hippocampus and entorhinal cortex are required for NOR. Fe+Cu animals had more severe impairments on discrimination and spatial tasks than LW, Fe, or Cu groups alone, which is in contrast to previous findings where Cu remediates impairments caused by Zn. This study showed that it is the balance of metals, not just an overload of a specific metal, which cause harm or not.

CHAPTER ONE: INTRODUCTION

Alzheimer's disease (AD) is the primary cause of dementia and the sixth leading cause of death in the United States (CDC, 2011). Age-related diseases and disorders are becoming more prevalent than ever before simply because people are living longer. By 2030, roughly 20 percent of the U.S. population (71M) will be considered senior citizens. The general population has access to a vast amount of information which supports the idea that dietary enrichment through supplementation (e.g., vitamins and minerals; enhanced bread, juices, milk) is beneficial, and from that arises the idea that there can be no harm in too much supplementation. Dietary supplements are regularly taken by fifty-two percent of adults, with an increasing proportion (63%) after the age of 60 (Radimer et al., 2004). However, for individuals with certain neurodegenerative brain disorders over supplementation of certain dietary compounds could result in increasingly rapid expression and progression of the disorders. In addition to intentional supplementation there is incidental exposure (e.g., environmental exposure, tap water) that may cause an additional increase in levels of these minerals in the body. For example, there are measurable levels of copper in the drinking water as a result of copper plumbing (Dietrich et al., 2004). Another example of increased exposure levels is the use of medications that contain high levels of metals, including the treatment for age-related

macular degeneration with high levels of zinc (AREDS, 2002), and excess zinc in denture cream (Hedera, Peltier, Fink, Wilcock, London, Brewer, 2009).

Within the healthy brain neocortical parenchyma, the transition metal ions, copper, iron, and zinc, are maintained at high concentrations; however, these transition metal ions are measured in increased concentrations in the AD-affected brain. Stores of iron and copper are necessary during early and reproductive years (Brewer, 2006). Conversely, with age the role of these metals changes and increased levels can be more detrimental, particularly in the AD brain. Iron and copper are two essential trace metals which are required by the human physiological system. Iron is essential to oxygen transport (Steinberg, Forget, Higgs, Nagel, 2001); vital to synthesis pathways of dopamine, serotonin, and catecholamines (Youdim, 1990); and other essential brain functioning processes (Berg & Youdim, 2006). Copper is essential for enzymes that are important in neurobiological functioning (tyrosinase, ceruloplasm, cytochrome *c* oxidase, dopamine β hydroxylase (Bush, 2000); required in cellular metabolic functions in the central nervous system (mitochondrial respiration, free radical defense) (Lutsenko, Bhattacharjee, Hubbard, 2010; Peña, Lee, Thiele, 1999); and is important in myelination, as a loss of myelin is associated with a copper deficiency.

Amyloid-beta (Aβ) aggregation has been proposed as a critical event in the etiology of AD (Hardy & Allsop, 1991; Joachim & Selkoe, 1992) and certain metals, such as iron, zinc, copper, and aluminum have been found to significantly accelerate the rate of aggregation of Aβ in physiological concentrations, *in vitro* (Mantyh et al., 1993; Esler, Stimson, Jennings, Ghilardi, Mantyh, Maggio, 1996). Iron (Fe(III)), copper

(Cu(II)), zinc (Zn(II)), and aluminum (Al(III)) are colocalized in significant concentrations with the pathological hallmark of AD, amyloid-beta or senile plaques (House, Collingwood, Khan, Korchazkina, Berthon, Exley, 2004). These metals are found in the surrounding tissue in human AD (Falangola, Lee, Nixon, Duff, Helpern, 2005; Lovell, Robertson, Teesdale, Campbell, Markesbery, 1999) and in transgenic AD mouse models (Maynard, Bush, Masters, Cappai, Li, 2005). Copper and zinc directly bind to the amyloid-β in plaques while iron is found to be concentrated in the area surrounding amyloid plaques (Barnham & Bush, 2008; Dong et al., 2003; Duce et al., 2010).

Even extremely small changes in free or exchangeable copper concentration may have an impact on Aβ solubility *in vivo* (Atwood et al., 2000). Age-dependent formation of amyloid pathology could be amplified due to the involvement of increased levels of copper, iron, and possibly synaptic zinc (Maynard et al., 2005). Findings on changes in copper levels in the brain are conflicting as levels have been shown to increase in the aging brain (Morita, Kimura, Itokawa, 1994; Loeffler et al., 1996; Maynard et al., 2002), whereas in the AD brain there appears to be a decrease in free copper ions (Wender, Szczech, Hoffman, Hilczer, 1992) and total brain copper (Diebel, Ehmann, Markesberr, 1996) A resulting copper deficiency is present in the brains of AD patients (Magaki, Raghavan, Mueller, Oberg, Vinters, Kirsch, 2007) and transgenic mice (Maynard et al., 2002; Bayer et al., 2003; Phinney et al., 2003). In the neocortex, levels of iron are shown to increase in the AD affected brain, while there is a decrease of copper (Barnham & Bush, 2008).

In human AD brain tissue, iron can be found bound specifically to senile plaques and absent in the surrounding glial cells rich in iron binding proteins, thus signifying the iron attached to lesions could be different than normal iron (Smith, Harris, Sayre, Perry, 1997). A significant increase in brain iron levels with age has been repeatedly shown, in both humans and mice (Thomas, Boyko, Anthony, Burger, 1993; Bartzokis, Beckson, Hance, Marx, Foster, Marder, 1997; Zecca et al., 2001; Maynard et al., 2002). Iron appears to play a role in APP and Aβ homeostasis. Increase in iron levels can cause a significant increase in intracellular APP, and APP levels decrease when cells are treated with deferroxamine (Df; an iron chelator) (Rogers et al., 2002). In addition to the role iron plays in aggregation of amyloid, high levels of iron also may be likely to promote the production of the amyloidogenic pathway due to down-regulation of furin activity thereby reducing α-secretase activity (Silvestri & Camaschella, 2008; Altamura & Muckenthaler, 2009). The role of copper is trickier in APP cleavage. Injections of furin enhance α-secretase in a transgenic mouse model of AD and furin mRNA levels are reduced overall in the AD brain (Hwang et al., 2006).

While copper is more likely to promote α-secretase cleavage and the non-amyloidogenic pathway (Borchart, Camakaris, Cappai, Masters, Beyreuther, Multhaup, 1999), it has a high affinity to bind to Aβ after cleavage resulting in free radical production (Rivera-Mancía, Pérez-Neri, Ríos, Tristán-López, Rivera-Espinosa, Montes, 2010). There appears to be a fine line for whether copper may be beneficial in preventing Aβ from forming, or becoming, more harmful once it has already developed. In transgenic animal models of AD, which result in an over-expression of APP, a

significantly lower level of copper is found in the brain (Maynard et al., 2002; Phinney et al., 2003).

This study examined the effects of trace metals on the TgCRND8 mouse model which is a double mutant Swedish (KM670/671NL) plus Indiana (V717F) mouse. It expresses the human APP 695 transgene (Chishti et al., 2001), and is also referred to as a Westaway mouse. The TgCRND8 mice begin to show deficits of reference memory at 11 weeks, correlating with the presence of amyloid at 3 months and the appearance of dense core plaques and neuritic pathology at 5 months of age (Chishti et al., 2001). Hyde et al. (2005) further classified age-progressive behavioral changes of CRND8 mice, showing changes tied to age-related neuropathology. There were no age differences in the Y-maze; however, MWM (Morris water maze) impairments were present in early and late plaque Tg mice; while pre-plaque Tg mice showed normal MWM spatial learning compared with Wt mice (Hyde et al., 2005). Standard TgCRND8 animals show NOR (novel object recognition) deficits but treatment with leptin, a long-term potentiation (LTP) enhancer, was able to compensate for these disease modulated deficits and allow for "remembering" (Greco et al., 2010). These findings correlate with data that Aβ40 and Aβ42 increase with age (Hyde et al., 2005).

The APP2576 mouse, which carries the Swedish mutation alone (APP_{SW}), shows more delayed cognitive deficits of 3 to 15 months (Hsiao Ashe, 2001). Studies showed resulting cognitive deficits at 18-21 months in the MWM (Railey, Groeber, Flinn, 2011) and NOR (Groeber, 2009). APP_{SW} transgenic animals show sensorimotor and cognitive changes which are progressive (3 months to 9 months) and gender-dependent (King,

Arendash, Crawford, Sterk, Menendez, Mullan, 1999). Notably these animals show impairment in the circular platform (Barnes Maze) and MWM retention and visible platform. Transgenic males showed increasing activity over controls in the open field and Y-maze tasks (King et al., 1999).

Previous studies of enhanced metal water in the Tg2576 model have shown that long-term dietary supplementation of zinc-enhanced water showed impairments in the spatial MWM task (Linkous, Adlard, Wanschura, Conko, Flinn, 2009) and those who received supplementation of zinc plus copper-enhanced water showed significantly less impairment (Railey et al., 2011). Enhancement with copper thus shows remediation of the deficits caused by dietary zinc-supplementation in AD-type transgenic animals. Tg2576 animals raised on iron-enhanced water showed similar deficits in the MWM to those raised on zinc-enhanced water (Railey et al., 2011). Tg2576 mice on zinc- and iron-enhanced water also showed impairments in the NOR task, with decreased NOR as well as decreased sniffing time (Groeber, 2009). Linkous et al. (2009) conducted a study using both Tg2576 and TgCRND8 mice given dietary zinc supplementation. Wild-type and all Tg mice showed impairment in spatial memory. Transgenic animals given zinc supplementation showed the greatest impairments of all subjects, regardless of transgenic strain (Linkous et al., 2009). These findings raise the question of whether copper could remediate the negative effects caused by iron, as it does for zinc.

One of the less discussed risk factors for Alzheimer's is gender; women, especially in the oldest old group (85 years of age and older), are at a higher risk for AD than men (Schmidt et al., 2008; Yaffe et al., 2011). Women also appear to be more

susceptible to cognitive deficits during the progression of the disease as seen through a larger cognitive drop from a healthy to an AD-diagnosis (Chapman et al., 2011). Cognitively normal females perform better on semantic and verbal tasks than men (Chapman et al., 2011); however, AD related cognitive impairments appear to reverse this advantage (McPherson, Black, Buckwalter, Cummings, 1999) and AD-diagnosed women then to have greater deficits on tasks which require personal knowledge, language ability, and episodic verbal memory (McPherson et al., 1999).

Post-menopausal hormone changes (i.e., estrogen and progesterone levels) may be responsible for these differences (Turner, 2001; Baum, 2005). Estrogen, as an antioxidant, can protect against neuronal damage caused by Aβ accumulation (Goodman, Bruce, Cheng, Mattson, 1996; Wicklgren, 1997) and with the maintenance of memory functions through interactions with acetylcholine in the hippocampus (Wickelgren, 1997). Consequently, a drop in estrogen levels in older, post-menopausal women could be one reason for the increased prevalence of AD in women. Estrogen replacement therapy (ERT) in women has been shown to delay the onset of AD (Paganini-Hill & Henderson, 1996). More amyloid is measured in the brains of senescent female transgenic mice than the brains of older male mice (Callahan, Lipinski, Bian, Durham, Pack, Walker, 2001). When amyloid plaques are formed, the Aβ levels increase more significantly in females compared to same-age male transgenic animals (Hirata-Fukae et al., 2008). Impairments in behavioral tasks can also be seen at earlier ages for female transgenic mice. APP_{SW} transgenic males showed a progressive impairment (from 3

months to 9 months) on the circular platform task, while transgenic females already showed severe impairments at 3 months (King et al, 1999).

Since past research has revealed that copper has the ability to remediate negative effects of zinc enhancement, it was hypothesized that copper would also remediate iron in spatial and discrimination tasks. To assess this, CRND8 mice were dosed with iron, copper and iron plus copper added to the drinking water for five months. They were evaluated in the NOR and the MWM to observe cognitive changes. The brain and liver of these animals are important for the understanding of the histopathological effects of supplementation with these metals; the brains were analyzed and the livers were preserved for future analysis. Groups of male and female mice were evaluated, as gender plays an important role in the level of cognitive impairment in AD. The results of this study are important for the future recommendation of supplementation of individuals with a genetic predisposition or diagnosis of AD.

CHAPTER TWO: MATERIALS AND METHODS

Mice

Subjects were transgenic (Tg) CRND8, or Westaway mice, with a double mutation (Swedish: KM670/671NL plus Indiana: V717F, which express the human amyloid precursor protein transgene). Wild-type (Wt) animals were of the C3HxC57 background. Breeding pairs of these mice were obtained from the University of Toronto. Mice were harem bred: one male was paired with three to four female mice for seven days. Prior to weaning, a small tail snip was collected from the pups for genotyping (Transnetyx, Cordova, TN). Pups with the desired genotypes (hAPP gene or Wt) were then weaned at postnatal day 21. After weaning, animals were group housed by sex in groups of three to four per cage. CRND8 mice show brain pathology at 5 months (Chishti et al., 2001) similar to APP2576 mice at 12 months (Hisao Ashe, 2001). They also show similar physical signs of aging such as graying and thinning fur and changes in gait. These changes are also observed in Wt mice. Each cage contained two igloos with one wheel attachment and one Nyla bone, and animals were handled daily. Mice were housed in a climate controlled 22°C ± 2°C environment with a 12:12 light/dark cycle and *ad libitum* access to food (Harland T.7102) 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.

Water

Water was prepared at George Mason University (GMU, Fairfax, VA) with tap water obtained from the main water supply of David King Hall and metal standards from SPEX CertiPrep Group (Metuchen, NJ). Treatment groups consisted of lab tap water; iron-enhanced water (10ppm $FeNO_3$); copper-enhanced water (0.2ppm $CuNO_3$); and iron- plus copper-enhanced water (10ppm $FeNO_3$ with 0.2ppm $CuNO_3$).

Table 1. Groups of Tested Animals.
A Transgenic; B Wild-types. Fe = iron water; Cu = copper water; Fe plus Cu = iron plus copper water; Lab = lab tap water.

A

N = 11	Female	TgCRND8 + Fe	Male	N = 9	
N = 13	Female	TgCRND8 + Cu	Male	N = 8	
N = 10	Female	TgCRND8 + Fe plus Cu	Male	N = 8	
N = 9	Female	TgCRND8 + Lab	Male	N = 8	Total = 76 mice

B

N = 13	Female	Wt + Fe	Male	N = 11	
N = 9	Female	Wt + Cu	Male	N = 9	
N = 12	Female	Wt + Fe plus Cu	Male	N = 9	
N = 13	Female	Wt + Lab	Male	N = 13	Total = 80 mice

Mice were raised on lab tap water from birth through 5 weeks of age and then transitioned onto supplemented water groups until sacrifice. Behavioral testing occurred at approximately 5 months of age, when animals had consumed metal-supplemented water for approximately 4 months; total time of consuming metal-supplemented water was approximately 5 months, including behavioral testing. Animals were tested using the novel object recognition (NOR) task and the Morris water maze (MWM) between five and six months of age. The pH of the enhanced metal water was tested after preparation to ensure proper pH for dietary consumption. Water was analyzed regularly using

inductively coupled plasma-optical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS, Reston, VA) to confirm metal content.

Experiment 1: Novel Object Recognition

Animals were tested at 5 months of age. The NOR box was a blue four-sided Plexiglas square box , 18 inches by 18 inches and 9.5 inches in height (Clever Sys., Inc., Reston, VA). Objects used were of similar size to the mice and attached to the NOR box using Velcro. Object Scan Behavior Analyzing system (TopScan, Clever Sys., Inc.) was used to track sniffing behavior by video recorded from a camera above the testing box. Sniffing behavior was measured through nose sniffing, when the animal points its nose directly toward the object.

Habituation: The animals were habituated for 5 days; during which they were placed in the NOR box and allowed to explore with varying paradigms each day. On Day 1, animals were placed in the box with cage mates with no objects, for 7 min. During Days 2, 3, 5, the animals were placed in the box individually with no objects, for 6 min. On Day 4, the animals were placed in the box individually with their standard object in the box, for 6 min. Varying durations of time spent in the box during habituation were used to prevent the animal from habituating to a specific time duration spent in the testing box. Following habituation, there were 4 trials of testing for novel object discrimination. During each trial animals were placed in the box with 2 objects, for 5 min.

Object Recognition: On Day 1, each animal was in the box for the first familiarization trial where they were presented with two copies of their standard object. In subsequent trials, the standard object and a novel object were present. Following the

initial familiarization trial, the animal was exposed to four more trials after delays of 15 min, 1 hr, 24 hrs, and 48 hrs. During each trial, the animal was presented with a new novel object. Between all trials, the NOR box and each object were cleaned with 70% ethanol. Water type, genetic type, object, and object location were counterbalanced.

Experiment 2: Morris Water Maze

Animals swam in a 4ft diameter pool surrounded on all sides by white curtains, each side with a distinctive cue to serve as external maze reference points. Coulbourn Instruments WaterMaze (Allentown , PA) software and computerized tracking system was used for recording and analysis of behavior. The cues on the four sides consisted of black cutout designs of various shapes and sizes. A transparent Plexiglas platform was submerged 1cm beneath the water surface; on "probe trials" the platform was completely submerged. The animals were placed in the pool at a different starting quadrant for each trial and the starting quadrant order was changed for each day of testing. The water was dyed with white, non-toxic tempera paint (Becker's School Supplies, Pennsauken, NJ) to help disguise the location of the submerged platform. The water temperature was maintained at 26$\pm$1°C.

Behavioral measures were latency, the number of platform position crossings on probe trials, the percentage of time spent in the target quadrant on probe trials, and thigmotaxia. Thigmotaxia is considered a measure of anxiety, and is defined as percent of time spent swimming the outermost 10% of the pool closest to the edge/wall.

Atlantis Platform: Each animal received three, 60s trials (A, B, C) per day for six days, with a 45s inter-trial interval. On every sixth trial (C trials on Days 2, 4, 6), the

mice were given a probe trial. One Day 7, animals were given a single 24 hour probe trial. During the probe trial the platform was made unavailable while the animal swam for 60s. The platform was raised after one minute, and if the animals had not yet found the platform they were gently guided to it and given 15s on the platform to observe the surroundings. This is considered a measure of spatial reference memory.

Visible Platform: Day 8 consisted of two visible platform trials during which the platform was marked to explicitly indicate its location. The platform was located on the opposite quadrant of the pool than in previous trials. These trials identify and eliminate any animals with sensory or motor deficits. During visible platform, mice were given two 60s trials, 45s apart.

Histological Analysis

After completion of behavioral testing, mice were sacrificed for the use of brain in histological analysis. Mice were anesthetized with carbon dioxide (CO_2; in cylinder) followed by decapitation. The brain and liver were immediately extracted. The brains were flash-frozen in dry ice, then stored in a -80°C freezer for longer term storage.

The left hemisphere of the brains were sectioned with a Leica CM3050 S cryostat in coronal sections ranging from 5 μm to 100 μm through the hippocampus, surrounding cortex, and basal ganglia; and placed on a normal glass slide for histological analysis. Five predetermined region of interests (ROI) were used for sectioning: ventral hippocampus (vHC; Bregma -3.16 mm), dorsal hippocampus (dHC; Bregma -1.94 mm), anterior hippocampus (aHC; Bregma -1.58 mm), basal ganglia 1 (BG1; Bregma -0.70 mm), and basal ganglia 2 (BG2; Bregma 0.74 mm) (Paxinos & Franklin, 2001). A

Congo red stain procedure was the primary histological analysis for the assessment of plaque load in the left hemisphere of the brain. The right hemisphere was preserved for analysis of amyloid (presence of and soluble vs. insoluble) and ceruloplasmin (CP) at a later date. Livers were preserved, fixed in 10% neutral buffered formalin (NBF) and paraffin embedded, for future histological analysis of iron load (Perl's Prussian blue) and copper load (rhodanine).

Congo Red

Congo red stain was used to determine the amount of amyloid plaque deposits in the tissue sections (Putchler's Modification; Florida State University College of Medicine, 2007). An Olympus BX51 fluorescent/polarizing microscope was used to view stained sections. ImageJ (National Institutes of Health, 2013) was used to quantify the images and the presence of amyloid and was used to calculate area and pixel value statistics of user-defined selections. A normalized score was created by defining the spatial scale of each brain slice image so that pixels were converted into millimeters.

CHAPTER THREE: RESULTS

Experiment 1: Novel Object Recognition

A significant difference in object recognition was found between CRND8 and Wt mice across all trials ($F_{(1,155)}$= 6.37, p=0.013). A significant sex difference was seen, females showed better object recognition than males ($F_{(1,155)}$=3.93, p=0.049; Figure 1). Further analysis showed a significant difference between sexes for Wt animals ($F_{(1,84)}$= 5.77, p=0.018), but not for Tg. There was also a statistically significant difference between Tg and Wt females ($F_{(1,87)}$= 7.36, p=0.008), Wt females scored better than Tg (Figure 1). Thus the Wt females performed significantly better than all other groups, which did not differ significantly from each other.

No overall significant difference between water groups was observed ($F_{(3,155)}$= 1.28, p=0.283). However, in Tg animals metal enhancement did affect performance in the object recognition task ($F_{(3,71)}$= 3.22, p=0.028; Figure 2). Post-hoc tests showed that Tg LW animals had statistically higher (p<.05) levels of object recognition than Tg Fe+Cu.

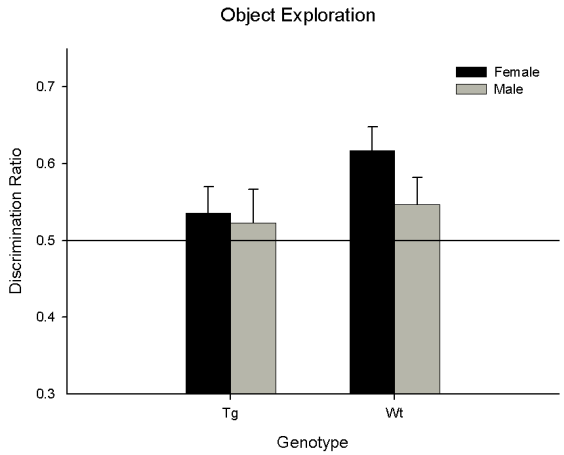


Figure 1. Object Exploration by Genotype and Sex.
Females showed better overall object recognition than males (p<0.05). There was no significant difference between Tg male and females, but Wt females did show more object recognition that Wt males (p=0.018). (Discrimination Ratio = novel object sniffing / (novel sniffing + standard sniffing)).

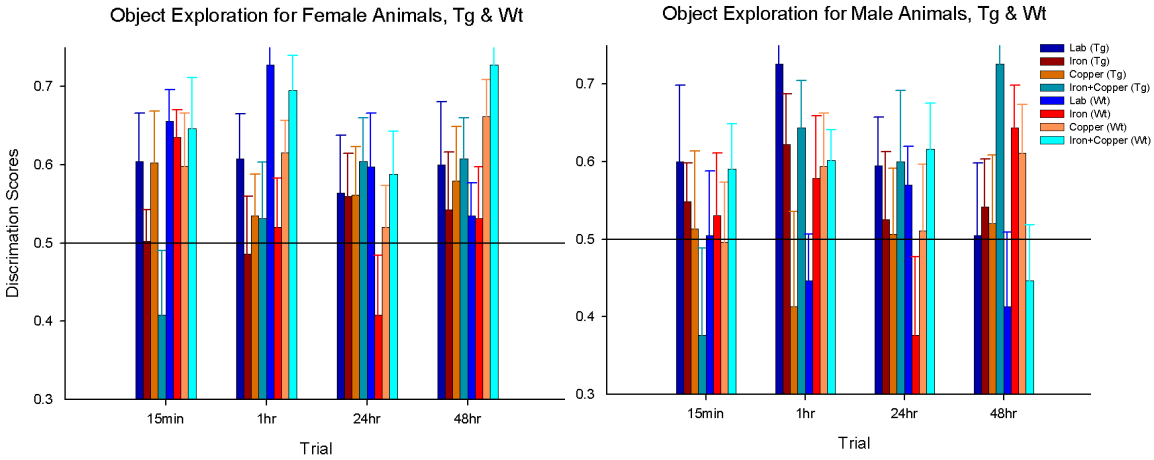


Figure 2. Object Exploration Across Trials, by Water Groups.
There was no overall significance of water groups. Metal enhancement does affect performance in Tg animals (p<0.05), and Tg LW animals has more object recognition than Tg Fe+Cu (p<0.05). (Discrimination Ratio = novel object sniffing / (novel sniffing + standard sniffing)).

Experiment 2: Morris Water Maze

There was a significant difference in latency across all days ($F_{(5,153)}$=3.234, p=0.007) by genotype where Wt animals showed larger decreases than Tg animals. Both Wt (p<0.001) and Tg (p<0.01) animals did show significant decreases in latency

(Figure 3). There was also a significant effect of sex, males showed significantly decreased latency compared to female animals ($F_{(1,153)}$=4.700, p=0.032; Figure 4) for both genotypes. There was no significant overall effect of water group on latency ($F_{(3,153)}$=1.930, p=0.127; Figure 5). However, post-hoc tests showed that Wt LW animals had significantly shorter ($p \leq 0.05$) latency than Wt Fe+Cu.

All animals located the visible platform to meet criteria for visible platform trials. No significant differences were found in latency during the visible trials for any of the groups indicating that there were no motor or visual deficits.

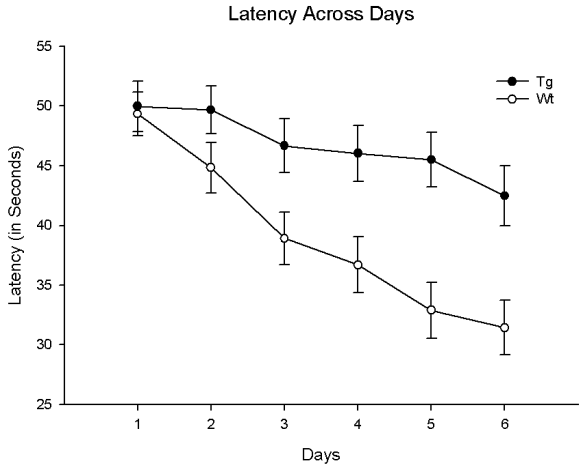


Figure 3. MWM Latency across Days 1-6, by Genotype.
Tg mice had significantly longer escape latencies than Wt mice across all days of testing (p<0.001).

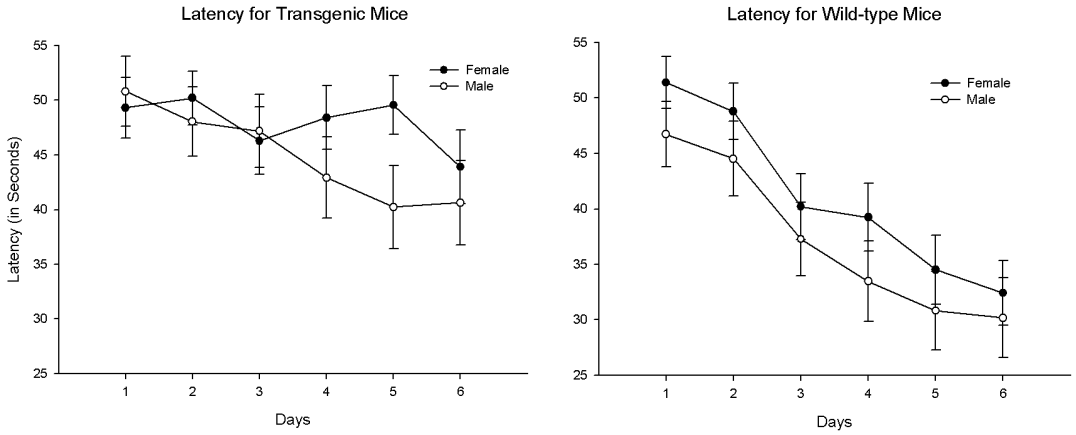


Figure 4. MWM Latency across Days 1-6, by Sex for Tg and Wt Mice.
There was a significant difference in escape latency between the male and female animals, regardless of genotype (p<0.05).

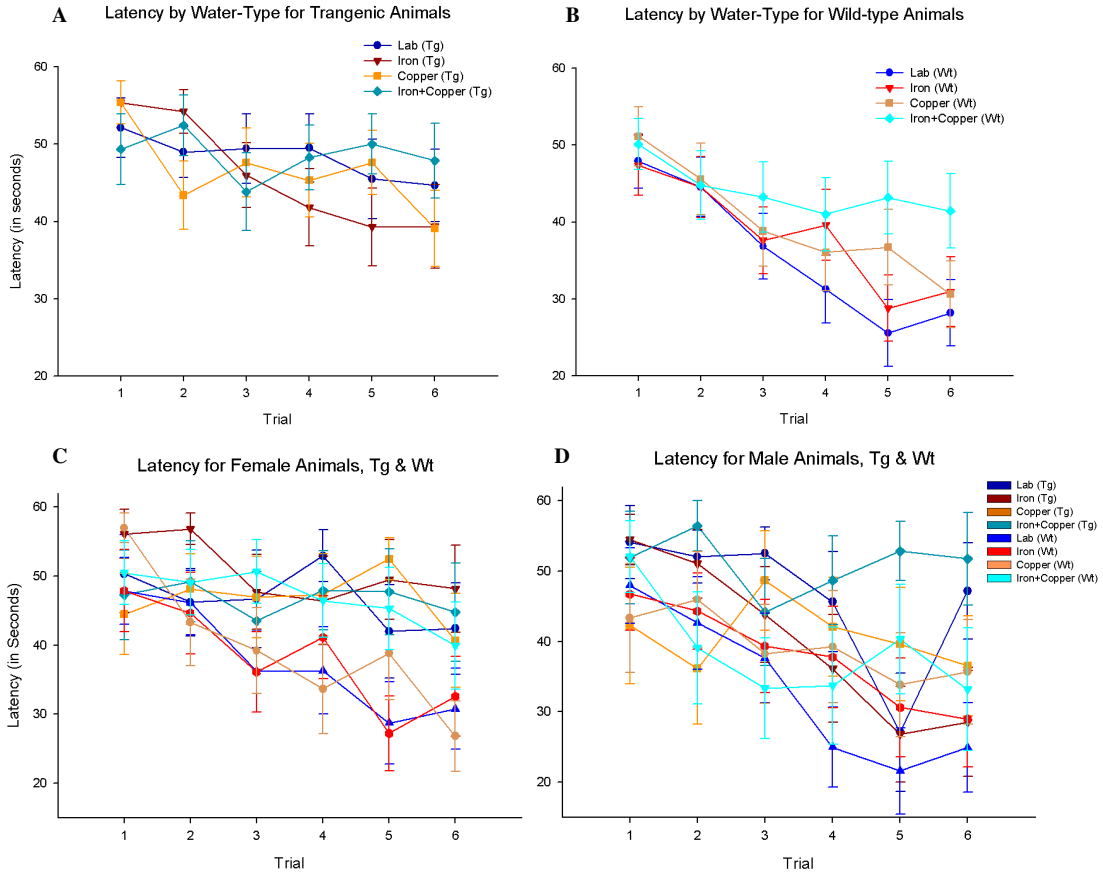


Figure 5. Latency across Days 1-6, by Water Group. Wt mice had shorter latencies compared to CRND8 mice, with no overall water effect.
A. Wt mice only; **B.** Tg mice only; **C.** Female mice only; **D.** Male mice only. There were no significant effects of water group, but Tg mice had longer escape latency times than Wt animals. Wt LW animals had shorter latencies than Wt Fe+Cu ($p \leq 0.05$).

Tg animals spent significantly less time in the platform quadrant than Wt animals ($F_{(1,153)}=5.289$, $p=0.023$). There was not a significant sex effect or water group effect, but there was a water x sex interaction ($F_{(3,153)}=2.417$, $p=0.069$), a water x genotype interaction ($F_{(3,153)}=3491$, $p=0.017$), and a trial x water interaction ($F_{(6,306)}=2.701$, $p=0.014$) (Figure 6A). There were no significant effects for genotype, sex, or water for platform crossings during probe trials (Figure 6B).

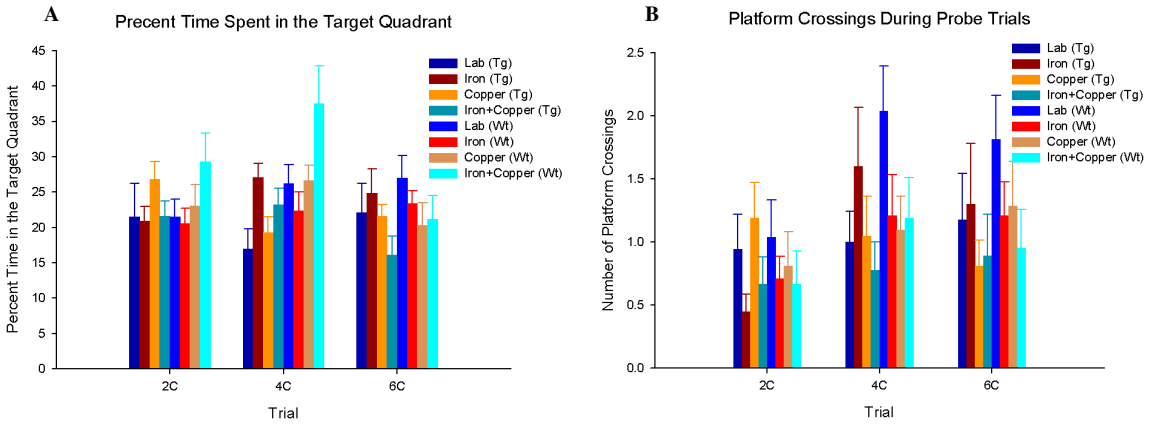


Figure 6. Percent Time in Target Quadrant and Number of Platform Crossings for Probe Trials.
CRND8 mice showed decreased time in the platform quadrant, but did not differ from Wt mice in platform crossings. **A.** Percent time in target quadrant during MWM probe trial Days 2,4,6. Tg animals spent significantly less time in the platform quadrant than Wt mice ($p<0.05$), but there were no significant effects of sex or water group. **B.** Platform crossings of platform location in target quadrant during MWM probe trials Days 2,4,6. There was no significant effect of genotype, sex, or water group.

There was a significant decrease in thigmotaxia for probe trials across days ($F_{(2,306)}$=7.2644, p=0.001), but no significant main effect of genotype. There was a significant effect of sex ($F_{(1,153)}$=6.724, p=0.010) and water ($F_{(1,153)}$=7.908, p<0.001). Post-hoc comparisons indicated that LW and Fe-treated animals spent significantly more time on the edge of the pool than Cu-treated animals. Additionally, significant interactions between water group x genotype ($F_{(3,153)}$=3.776, p=0.012) and water group x genotype x sex ($F_{(3,153)}$=3.066, p=0.030) were obtained.

Histological Analysis

All animals examined were Tg as plaque formation should only occur in Tg-AD animals. There was no overall significant difference for water groups; however, planned comparisons showed that differences occurred among specific water groups. LW animals had significantly more plaques than Cu-treated animals ($F_{(1,13)}$=6.522, p=0.024) and Fe+Cu-treated animals also showed a trend for more plaques than Cu-treated animals ($F_{(1,14)}$=3.892, p=0.069; Figure 7A), when males and females were analyzed together. Female animals also showed a trend for significance across water groups ($F_{(3,15)}$=2.595, p=0.091). Post-hoc comparisons indicated that female Fe+Cu animals had a trend for more plaques than female Cu animals (p<0.10). There were no significant differences for male animals. Therefore, the water deficits were due largely to the females.

When considering ROI, there was no overall sex or water difference. Female animals of all water groups showed a trend for significance of plaque area across ROIs ($F_{(4,60)}$=2.062, p=0.074; Figure 7B,C), in general there was a higher plaque burden in the hippocampus than the basal ganglia.

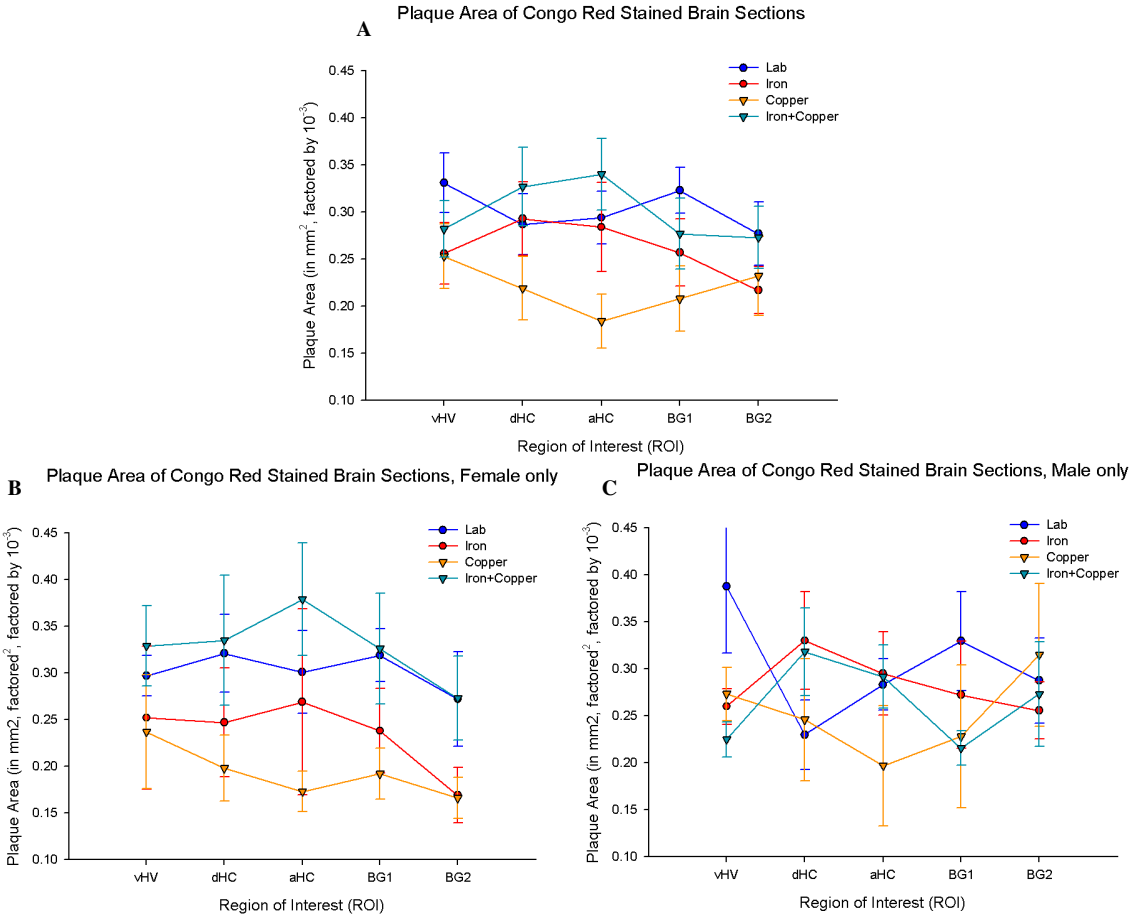


Figure 7. Plaque Load Levels.
A. All animals; **B.** females only; **C.** males only. LW animals had more plaques than Cu animals ($p<0.05$). Fe+Cu animals showed a trend for more plaques than Cu animals ($p=0.069$). Females showed a trend for plaque levels across water groups ($p=0.074$).

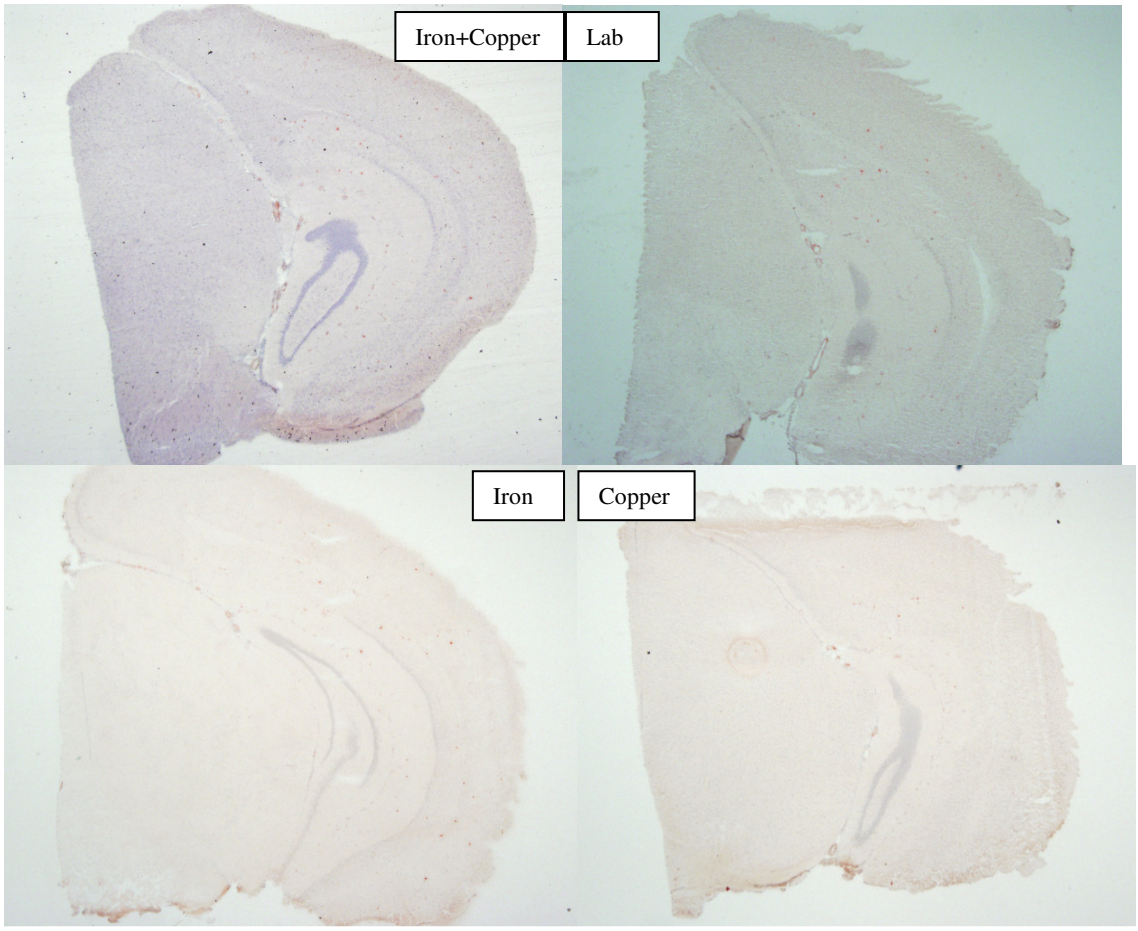


Figure 8. Congo Red Images with Plaques, from ROI 1 Ventral Hippocampus (vHC).

CHAPTER FOUR: DISCUSSION

This study aimed to expand the understanding of the role iron and copper play in behavioral outcomes and pathology of Alzheimer's disease. Previous studies have shown that excess metal, specifically zinc and iron, in the diet of transgenic AD mice can cause learning and memory impairments (Railey et al., 2010, 2011). The literature has been less clear on the effects of copper in a transgenic model of AD. Copper studies are often combined with those that also examine the effects of cholesterol. Sparks & Schreurs (2003) determined that copper increased impairment in a rabbit model of AD, on rabbits fed a high cholesterol diet. Contrasting research shows that copper can remediate the negative learning and memory effects seen with dietary enhancement of zinc (Railey et al., 2010, 2011). This begs the question of whether copper causes a similar remediation of the negative effects caused by dietary iron overload.

Important to the study was to determine the role copper would play in iron supplementation of behavior. This study has shown that it is the balance of metals, not just an overload of a specific metal, which causes harm or not. Effects may differ in Wt and Tg mice. In NOR, Tg Fe+Cu-treated animals showed decreased object recognition compared to Tg LW animals, while Wt Fe+Cu-treated animals performed well. In MWM, the Fe+Cu-treated animals took the longest time to find the platform, and both the Wt and Tg Fe+Cu-treated animals failed to find the platform. This indicates that even

in normal animals, high levels of Fe+Cu is can have negative effects. In contrast, Wt Fe-treated and Cu-treated animals show similar learning curves compared to Wt LW. Only the Fe+Cu-treated animals did not have a decrease in latency and were significantly different from the LW, who were the fastest. Fe-treated animals started poorly, but ended at a similar latency (Wt) or a decreased latency (Tg) than LW animals. This differs from previous findings (Railey et al., 2011), but is possibly due to the different strain of the mouse model (CRND8 in the current study, Tg2576 in the previous study) and younger age of the CRND8 mice. Although we hypothesized that Cu would enhance performance, particularly in animals suffering from negative behavioral effects of Fe supplementation, this is not what our study found. In contrast to findings that Cu helps Zn overload, Cu is not beneficial in Fe supplementation, and Fe+Cu animals generally performed the worse.

Our findings indicate that females and males perform differently in behavioral tasks dependent upon the type of task. The level of novel object recognition differed between male and female Wt mice, but not Tg. This indicates that female Wt mice are better at this task than male Wt mice; however, once these animals develop signs of AD the difference between the sexes disappears. This is comparable to human sex differences in AD which show that women are more susceptible to cognitive deficits during the progression of AD. Cognitively intact women perform better (semantic and verbal tasks) than men (Chapman et al., 2011), but with AD a larger cognitive drop occurs for women (McPherson, Black, Buckwalter, Cummings, 1999).

In contrast, in MWM, males swam faster to the platform than females, and spent less time around the edge of the pool. This was the most pronounced in Tg males

compared to Tg females. Male animals showed that they had better spatial memory allowing them to swim more directly to the platform and spend less time around the edge of the pool as a search technique or sign of anxiety. We hypothesized that female animals would perform worse on all behavioral tasks due to the cognitive effect of AD, what our study found was that this is the case in the MWM for Tg animals, but male and female Tg animals perform similarly in NOR.

An interesting finding in the histological analysis was that Fe+Cu-treated and LW Tg animals had the largest plaque burden. It might be expected that LW animals would have a low plaque burden; however, the finding that LW had higher plaque burden than metal supplemented animals (Fe) has been seen in a previous study (Groeber, 2009). Females have more plaque area, similar to what is seen in senescent female mice with larger amounts of amyloid found in the brains compared to age-matched males (Callahan, Lipinski, Bian, Durham, Pack, Walker, 2001; Hirata-Fukae et al., 2008). Female Fe+Cu animals show the highest plaque area levels, followed by LW, Fe, and Cu. In males, differences in plaque burden due to supplementation are less clearly defined. But here again, Fe+Cu has a more negative impact that Fe alone. Our hypothesis of plaque accumulation is based on the idea that supplementing with additional metals provides the building blocks for plaques in the AD-brain. We hypothesized that LW animals would have the lowest plaque accumulation, as there is no additional metal, but these animals and Fe+Cu had the highest level of accumulation. We did predict the low level of accumulation for Cu animals, although it was unexpected to see animals with Fe+Cu have a higher plaque accumulation than those with Fe alone.

Plaque data correlates with MWM and NOR outcomes for Tg Fe+Cu animals; these animals perform poorly on both tasks and have a high level of plaques. However, although Tg Fe+Cu animals have the lowest levels of object recognition, the Tg LW perform well on NOR and Tg Cu, with the lowest number of plaques, did not. In MWM, the Tg LW and Tg Fe+Cu animals had the longest latencies and the least decrease in latency. Cu animals showed good performance in MWM with some of the fastest decreasing latencies, and also had the lowest plaque levels. This indicates that measurement of plaque burden may be not be a predictive measure of NOR, but it may be predictive of MWM and it may be due to location of plaque accumulation. This is possibly due to the brain regions required for the completion of the task; MWM depends on the hippocampus, while the hippocampus and entorhinal cortex are required for NOR. The plaque burden was assessed on an entire slice of hippocampal region; however, these findings indicate that it would be more appropriate to make a distinction between the hippocampus and the entorhinal regions. In the plaque analysis it could be seen that there were more plaques in the hippocampus and adjacent cortical regions and less in the ventral and entorhinal regions (Figure 8). This is of importance as NOR requires the entorhinal cortex in addition to the hippocampus.

CRND8 animals were impaired on all assessed measures of learning and memory compared to Wt animals. In NOR, the CRND8 animals were less likely to show preference for the novel object over the familiar, displaying an impaired ability in object recognition. In MWM, the CRND8 animals were slower to find the platform and spent less time in the target quadrant than the Wt animals. The CRND8 animals were not able

to recall the location of the platform or use the cues as a search strategy to find the target quadrant. These behavioral tasks and specific paradigms are therefore appropriate for examination of behavioral outcomes of AD, specifically this double-mutant APP model which involves a rapid rate of aging with behavioral and pathological markers of AD by 5 months.

There was a difference between male and female animals depending on the task on which they were being assessed. Female Wt performed better on NOR, a non-spatial discrimination task, where males (Wt and Tg) performed better on MWM, a spatial task. These findings are in agreement with human sex differences, which hold that in general men have better spatial memory and women have better episodic memory. The brain regions required for these specific types of learning and memory may play an important role in understanding why these sex differences occur.

Both iron and copper are required in the human physiological system for many essential functions (Wigglesworth and Baum, 1988; Youdim, 1990; Bush, 2000; Lutsenko, Bhattacharjee, Hubbard, 2010). However, in the aging brain, the concentration of these metals increases (Maynard et al., 2002) and in the AD-brain these metals are known to play a role by binding to the plaques and surrounding tissue (Lovell et al., 1998; Dong et al., 2003; House et al., 2004).

Iron is known to be associated with the pathogensis of several neurodegenerative disorders, such as AD, Parkinson's disease, and neurodegeneration with brain iron accumulation (NBIA). In the aging brain ferritin, a storage protein of iron in the brain, is able to store larger amounts of iron. This leads to an increased risk of iron-induced

peroxidative damage due to this increase in iron (Beard et al., 1993). The level of iron is important because increased iron can lead to an increase in intracellular APP, and cleavage of APP by α-secretase further leading to increased Aβ production.

The role of copper in AD has been discussed extensively. Prior to cleavage of APP, copper may promote the non-amyloidogenic pathway (Borchardt et al., 1999); however, after cleavage towards the amyloidogenic pathway Aβ has a high affinity for copper (Rivera-Mancía et al., 2010). Additionally, a decrease in intracellular copper can lead to decreased APP expression (Bellingham et al., 2004) and increased processing of APP to the amyloidogenic pathway (Cater et al., 2008). The important question about copper in AD may not be whether there should be more or less copper but when should an exposure of increased copper levels occur, and could it be beneficial if the exposure was at the appropriate time. Perhaps if copper supplementation is given earlier in life to protect against development of the amyloidogenic pathway, there might be a treatment plan with dietary copper.

The most interesting behavioral finding of water group in this study was the drastic impairment of animals on Fe+Cu-enhanced water. This could be due to the interaction iron and copper play in the body. Ceruloplasmin (CP) is a multicopper ferroxidase enzyme that is the major copper carrying protein in the blood and plays a role in the metabolism of iron. In AD patients the amount of CP is normal or increased, but the activity of each CP enzyme is decreased (Boll, Alcarez-Zubeldia, Montes, Rios, 2008). CP plays an important role in the oxidation of ferrous to ferric iron, and may be required to prevent a dangerous build-up of ferrous iron (Reeves & DeMars, 2004;

Yangisawaa et al., 2009). This oxidation reaction also allows for CP to bind to transferrin (Reeves & DeMars, 2004; Yangisawaa et al., 2009). CP is also important in copper regulation in the liver (Squitti et al., 2007).

The detrimental effects caused by Fe+Cu supplementation may be explained by the overload of both copper and iron in the system allowing for multiple failures. Not only is there a toxic build-up of these two metals, both of which have been shown to cause APP to form Aβ, but a failure of the enzymatic activity of CP could occur; thus preventing the oxidation of ferrous iron and the transport of both iron and copper away from the brain to other physiological systems, such as the liver.

The results of this study indicate that there are sex differences across varying behavioral tasks of learning and memory. This emphasizes the fact that it is important to study both sexes for behavioral paradigms and not only one sex or to group both sexes together. Further examination of the role copper plays in AD and APP processing is necessary as also indicated by these results as Fe+Cu animals had more severe impairments on discrimination and spatial tasks than LW, Fe, or Cu groups alone. Moreover, it is important to examine the overall metal balance in the brain. It is also important to look at metal combinations in *in vitro* work. While previous studies have shown that Cu remediated impairing effects caused by Zn supplementation, this study shows that the same level of Cu, when combined with Fe, does not remediate impairments but causes more severe limitations on learning and memory. How should the question of whether copper supplementation is good or bad be answered; it depends on the presence or absence and the quantity of all metals in the brain.

APPENDIX A: LITERATURE REVIEW FROM DISSERTATION PROPOSAL

Background and Significance

Alzheimer's disease (AD) is the primary cause of dementia and the sixth leading cause of death in the United States (Center for Disease Control and Prevention [CDC], 2011). There are 500,000 people newly diagnosed with AD each year with a current estimate of 5.4 million individuals in the American population diagnosed with Alzheimer's disease overall. These numbers are expected to increase exponentially and the population of individuals with AD could range from 11.3 to 16 million by the year 2050 (Herbert, Scherr, Bienias, Bennett, Evans, 2003; Alzheimer's Association, 2013). This disease has become not only one of the most deadly, but one the greatest medical, social, and economic challenges to the United States due to the impact on the individuals afflicted, their family and friends, and our nation's health care system (Alzheimer's Association, 2011). Age-related diseases and disorders are becoming more prevalent than ever before simply because people are living longer. By 2030, roughly 20 percent of the U.S. population (71M) will be considered senior citizens. This makes fields of research pertaining to diseases that afflict the elderly increasingly more important.

A diagnosis of early onset AD is generally genetically based; however, these cases represent only about five percent of the population. While AD diagnoses are predominantly late onset, these cases are the most difficult to determine the cause of the disease (Isik, 2010). Genetic bases are different in early versus late on-set AD; however, cellular changes are similar. With the intention of achieving a health benefit, there are ways in which many people are enriching their diets with supplements which could potentially be harmful and increase the risk of disease expression. Dietary supplements/vitamins and minerals, enhancement of all types of food and drinks (e.g., bread, juice, milk), and incidental exposure (e.g., environmental exposure, tap water) are all potential sources of overloading the human physiology in an attempt to be healthier. Recent studies have even begun to show measurable levels of copper appearing in the drinking water from copper plumbing (Dietrich et al., 2004). Furthermore, the elderly population which is already at-risk for Alzheimer's disease may be advised to use specific supplements or other medications that contain high levels of metals, such as the case for treatment of age-related macular degeneration with high levels of zinc (AREDS, 2001) and excess zinc in denture cream (Hedera et al., 2009).

Alzheimer's disease, APP & Amyloid-β, and Trace Metals

Alzheimer's disease is a polygenic neurodegenerative disease characterized by senile plaques, formed from amyloid-beta protein (Aβ), and neurofibrillary tangles, formed by tau protein. (This introduction and study will focus on the amyloid plaques.)

Iron and copper have been found in the amyloid plaques and surrounding tissue of the brains of patients diagnosed with Alzheimer's disease (AD) (Falangola, Lee, Nixon, Duff, Helpern, 2005; Lovell, Robertson, Teesdale, Campbell, Markesbery, 1998) and in transgenic AD mouse models (Maynard, Bush, Masters, Cappai & Li, 2005). Lovell et al. (1998) have provided evidence that, within the neuropil, levels of Zn^{2+}, Fe^{3+}, and Cu^{2+}, are significantly elevated compared with the non-AD neuropil; and also that these metal ions are significantly more concentrated within the core and periphery of amyloid plaque deposits.

Through the process of proteolysis, amyloid precursor protein (APP) transforms into the amyloid beta (Aβ) peptide that is deposited into the brain in cases of AD (Clements, Allsop, Walsh, Williams, 1996). Deposits of Aβ are found in the form of senile plaques, or dense extracellular deposits of β-pleated sheet fibrils, diffuse plaques, and cerebrovascular amyloid in the neuropil (Atwood et al., 1998). Cleavage of APP will lead to one of two competing pathways: (1) the amyloidogenic pathway which produces Aβ after two proteases, β-secretase and γ-secretase, cleave the APP protein; or (2) the non-amyloidogenic pathway which prevents Aβ production after cleavage by α-secretase within the APP sequence (Selkoe, 1998; Figure 9).

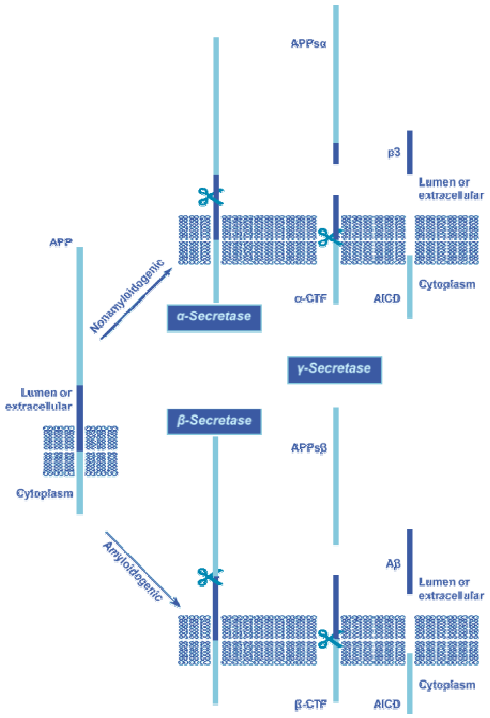


Figure 9. Proteolytic processing of the amyloid precursor protein (APP) by secretases.
(European Iron Club, 2007)

Aβ aggregation has been proposed as a critical event in the etiology of AD (Hardy and Allsop, 1991; Joachim and Selkoe, 1992) and exposure to certain metals, such as aluminum, iron, zinc, and copper may play a role in this aggregation. Iron (Fe(III)), aluminum (Al(III)), copper (Cu(II)), and zinc (Zn(II)) are found to be colocalized in significant concentrations with the Aβ of senile plaques (House et al., 2004). Aluminum, iron, and zinc cations have also been found to significantly accelerate the rate of aggregation of Aβ, *in vitro*, in physiological concentrations (Mantyh et al., 1993; Esler et al., 1996).

There is an increase of α-secretase cleavage in the presence of copper, which can lead to an increase of the non-amyloidogenic APP fragment. Hence, copper appears to promote the non-amyloidogenic pathway of APP (Borchardt et al., 1999) However, Aβ has a high affinity for copper after cleavage via the amyloidogenic pathway (Rivera-Mancía et al., 2010). The binding of copper to Aβ increases the production of free radicals (Rivera-Mancía et al., 2010). There may be an increase in β-secretase in the presence of high levels of iron due to decreases in furin activity (Figure 10), thus high iron levels are more likely to promote the amyloidogenic pathway through the creation of APP (Silvestri & Camaschella, 2008; Figure 10).

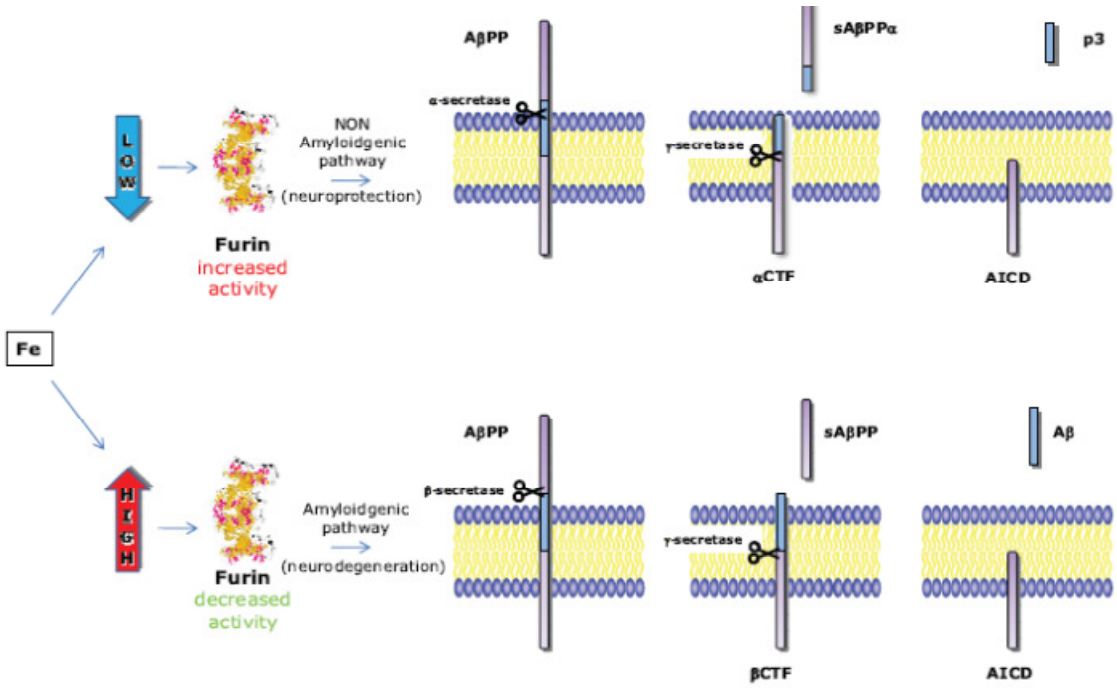


Figure 10. Iron (Fe), furin, and AβPP cleavage.
(Altamura & Muckenthaler, 2009)

Trace Metals and their Physiological Function in the Normal Brain

Iron and copper are two essential trace metals which, because they are biochemically functional metals, are required by the human physiological system, but only in small amounts. Trace metals are important to many essential activities of the body and brain, and in healthy individuals, consumption of a regularly balanced diet will meet the required intake of these metals. Strict regulation of metal movement across the blood-brain barrier leaves the brain metal content of a normal brain at a constant level (Barnham & Bush, 2008).

Iron is an essential nutrient to the brain serving as a component of numerous cellular enzymes and is involved in various levels of neurological activities. Iron is fundamental to oxygen transport as it is the central molecule in the oxygen transport structure and system. Hemoglobin, the oxygen-transport metalloprotein in red-blood cells, contains four heme groups which each include a bound iron ion (Steinberg, Forget, Higgs, Nagel, 2001). Iron is also vital to neurological activities of the brain including synthesis pathways of dopamine, serotonin, catecholamines (Youdim, 1990), and possibly γ-aminobutyric acid (GABA) (Hill, 1985) and myelin formation (Larkin and Rao, 1990). Iron is a necessary constituent for cytochrome oxidases, numerous enzymes in the citric acid cycle, ribonucleotide reductase, and NADPH reductase (Wigglesworth and Baum, 1988). Iron is essential to the correct functioning of the brain as it is a crucial cofactor for a number of biological processes, including gene expression, neuronal development, enzymatic reactions, heme, and electron transport (Berg & Youdim, 2006).

Copper (Cu^{2+}) is essential for the function of various enzymes (tyrosinase, ceruloplasm, cytochrome *c* oxidase, dopamine β hydroxylase) that are important in neurobiological functioning (Bush, 2000) and acts as a cofactor for antioxidant enzymes (Peña, Lee, Thiele, 1999). General cellular metabolic functions, such as (mitochondrial) respiration and free radical defense, require copper in the central nervous system (CNS) (Lutsenko, Bhattacharjee, Hubbard, 2010; Peña et al., 1999). Myelination of neurons also requires a balance of copper, as a loss of myelin is associated with a copper deficiency. Thus iron and copper are important for indispensable brain function including cellular respiration and energy production, enzyme function, neurotransmitter synthesis, and antioxidant function.

It is important to consider that stores of iron and copper are necessary during early and reproductive years and are beneficial to the younger adult for more positive health (Brewer, 2006). Conversely, with age the role of these metals changes and increased levels can be more detrimental, particularly in the AD brain. However, there is still a fine balance which must be maintained, as deficiency can be just as devastating.

Interaction of Trace Metals in Alzheimer's disease

Within the healthy brain neocortical parenchyma, the transition metal ions, copper, iron, and zinc, are maintained at high concentrations; however, these transition metal ions are measured in increased concentrations in the AD-affected brain. In the AD brain, these metal ions are also highly concentrated within amyloid plaque deposits. Overall, current research suggests that even extremely small changes in free or exchangeable copper concentration may have an impact on Aβ solubility *in vivo* (Atwood

et al., 2000). Age-dependent formation of amyloid pathology could be amplified due to the involvement of increased levels of copper, iron, and possibly synaptic zinc (Maynard et al., 2005). A significant increase in brain iron levels with age has been repeatedly shown, in both humans and mice (Thomas, Boyko, Anthony, Burger, 1993; Bartzokis et al., 1997; Martin, Ye, Allen, 1998; Zecca et al., 2001; Maynard et al., 2002). Findings on changes in copper levels in the brain are conflicting as levels have been shown to increase in the aging brain (Morita, Kimura, Itokawa, 1994; Loeffler et al., 1996; Maynard et al., 2002), whereas in the AD brain there appears to be a decrease in free copper ions (Wender, Szczech, Hoffmann, Hilczer, 1992) and total brain copper (Deibel et al., 1996).

As discussed, iron and copper are vital elements for the proper physiological functioning of the human body and proper brain functioning. It is also important to understand that both iron and copper can play an important role in the pathogenesis of multiple neurodegenerative diseases. In AD the balance of brain metals shifts and it is therefore important to understand the interaction of iron and copper with Aβ, as the key component of plaques, and APP (the longer protein which leads to the production of amyloid in AD). Aβ and APP are metalloproteins that are comprised of specific binding sites for zinc and copper. Copper and zinc directly bind to the amyloid-β in plaques while iron is found to be concentrated in the area surrounding amyloid plaques (Barnham & Bush, 2008; Dong et al., 2003; Duce et al., 2010). APP has been shown to act as a ferroxidase in neo-cortical cells, responsible for the removal of the oxidizing agent Fe^{2+}; however, zinc can inhibit this process (Duce et al., 2010). Failure to remove Fe^{2+} can lead to increases in neuronal death. In the neocortex, levels of iron are shown to increase in the AD affected brain, while there is a decrease of copper (Barnham & Bush, 2008). Over-production of soluble Aβ (the more toxic form of amyloid which can directly lead to neuronal death) cannot sufficiently explain Aβ precipitation, leading to the hypothesis that biochemical mechanisms which promote Aβ formation, in the non-soluble form, may be relevant to the pathogenesis of Alzheimer's disease (Bush et al., 1994).

An altered neuronal H^+ homeostasis may be seen in AD along with the appearance of amyloid deposits, representing an increased acidic state of the AD brain. Cerebral acidosis may complicate AD (Yates, Butterworth, Tennant, Gordon, 1990) and may be related to the inflammatory response seen in AD brain tissue (Griffen, Sheng, Mrak, 1997; Rogers and O'Barr, 1997). Iron and copper are redox-reactive metals which bind to amyloid and, through the Fenton reaction, form hydrogen peroxide (H_2O_2) generating a reactive hydroxyl radical. During the Fenton reaction, the amyloid-β peptide catalyses the reduction of Cu^{2+} to Cu^+ and Fe^{3+} to Fe^{2+} to form H_2O_2. The resulting free radical intensifies oxidative damage, oxidative stress, and neuronal death (Opazo et al., 2002; Huang et al., 2004; Khan, Dobson, Exley, 2006; Smith et al., 2010). Zn^{2+} and Cu^{2+} have the ability to induce 30% more Aβ aggregation than other bioessential metal ions including Fe^{3+}, with copper causing the most striking increase in Aβ aggregation (Atwood et al., 1998). These results reaffirm the importance of the Aβ:Cu^{2+} interactions to the pathophysiology of amyloid deposition (Cherney et al., 1999). The levels of several copper and iron regulatory and storage proteins are altered in the AD brain as well

(Basun, Forssell, Wetterberg, Winblad, 1991; Loeffler et al., 1996; Castellani, Smith, Nunmura, Harris, Perry, 1999; Maynard et al., 2005).

As changes in soluble Aβ levels occur only in the diseased brain, there must be a change which is specific to the composition and structure of the brain allowing the CSF to be exempt from this harmful increase. Soluble Aβ is not found to be increased in the cerebral spinal fluid (CSF) of AD patients demonstrating that there are other pathogenic mechanisms likely to be involved in the aggregation of Aβ in the brain. Could this be linked to the increases in copper, iron, and zinc in the CSF of AD patients? Not only can a significant increase (2.2 fold) in the concentration of copper in the CSF (Basun et al., 1991) be measured in AD patients, but also an increase in brain ceurloplasmin, a copper transport protein (Loeffler et al., 1994). CSF iron concentration has been reported to exceed the iron-binding capacity of transferrin present in the CSF (Bleijenberg, von Eijk, Leijnse, 1971). Transferrin is the protein responsible for delivering iron across the blood-brain barrier (Fisherman, Rubin, Handrahan, Connor, Fine, 1987; Partridge, Eisenberg, Yang, 1987).

Iron in AD

In human AD brain tissue, iron can be found bound specifically to senile plaques and absent in the surrounding glial cells rich in iron binding proteins, thus signifying the iron attached to lesions could be different than normal iron (Smith, Harris, Sayre, Perry, 1997). At the location of the lesion-bound iron, Smith et al. (1997) also demonstrated that there was redox activity, which is consistent with the idea that oxidative damage appears only at the site of AD lesions, such as plaques and tangles.

Dysfunctional regulation of the proteins responsible for regulation of iron storage and transport can cause an iron imbalance in the brain (Beard, Connor, Jones, 1993). In comparing normally aged brain tissue at comparable locations of AD brain tissue, transferrin concentration is found to be lower, ferritin levels are either unchanged or slightly decreased, and iron levels are higher (Connor, Boeshore, Benkovik, 1992a). This suggests that in the AD brain, there is increased storage of iron per mol of ferritin and decreased mobility of iron (even though as much as 4,500 atoms of iron can be stored in ferritin [Beard et al., 1993]). Therefore, the risk of iron-induced peroxidative damage is increased due to a boost in iron, as iron can readily be moved from ferritin by a reducing agent (Beard et al., 1993). Ferritin is an iron regulatory protein in the brain which has the potential to be responsible for the storage of one–third of brain iron. In human, rat, and mouse brains, several groups have reported that ferritin is predominantly contained in oligodendrocytes and microglial cells (Beard et al., 1993; Connor, Snyder, Beard, Fine, Mufson, 1992b; Connor & Benkovic, 1992). This has important implications for the effect of iron overload on neuronal development and communication. An excess of ferrous (Fe^{2+}) iron in the brain may lead to neuronal degeneration, further leading to an increase in Aβ plaque formation due to free radical generation in the presence of iron (Lovell et al., 1998). *In vitro* studies have shown that excess ferric (Fe^{3+}) iron also influences plaque formation through an accelerated rate of formation of more dense and tightly packed $A\beta_{42}$ fibrillary structure (House et al., 2004). Ferrous iron (Fe^{2+}) is the more dangerous state of iron because it is produced as a result of the Fenton reaction,

where ferric iron (Fe^{3+}) is reduced and results in free radial damage, in addition to its increased role in Aβ plaque formation.

Iron appears to play a role in APP and Aβ homeostasis. Increase in iron levels can cause a significant increase in intracellular APP, and APP levels decrease when cells are treated with deferroxamine (Df; an iron chelator) (Rogers et al., 2002). Pathways mediating ferritin translation (iron-response element [IRE] dependent) also control APP gene expression in relation to cellular iron levels (Rogers et al., 2002).

High levels of cellular iron may decrease rates of α-secretase, resulting in an increase in Aβ production. Iron has recently been tied to the furin molecule, a ubiquitous enzyme which is required for processes including α- and γ-secretases. It has been suggested that increased iron levels can down-regulate furin protein expression, thereby reducing α-secretase activity and resulting in Aβ production (Altamura & Muckenthaler, 2009; Silvestri & Camaschella, 2008). Injections of furin enhance α-secretase in a transgenic mouse model of AD and furin mRNA levels are reduced overall in the AD brain (Hwang et al., 2006).

Hallervorden-Spatz syndrome is an iron deposition and overload disorder. It is characterized by abnormal iron accumulation in the brain resulting in brain degeneration, and is also known as neurodegeneration with brain iron accumulation type1 (NBIA-1; Neumann et al., 2000). Patients show classic accumulation of brown pigmentation in perivascular brain structures, the neuropil, globus pallidus and substantia nigra pars reticulata (SNpr) due to exceedingly high iron deposition. Progressive dementia develops, including aphasia, amnesia, and agnosia, as well as increased motor dysfunction (Cooper, Rizzo, Jones, 2000).

Copper in AD

Although copper levels are increased within extracellular amyloid plaques (Lovell et al., 1998; Dong et al., 2003; House et al., 2004), an incongruent copper deficiency is present in the brains of AD patients (Magaki et al., 2007) and transgenic mice (Maynard et al., 2002; Bayer et al., 2003; Phinney et al., 2003). In transgenic animal models of AD, which result in an over-expression of APP, a significantly lower level of copper is found in the brain (Maynard et al., 2002; Phinney et al., 2003). APP and APLP2 (amyloid precursor-like protein) expression has been shown to alter copper homeostasis in the mouse cerebral cortex and liver by lowering copper levels, but not zinc or iron, compared to wild-type (Wt) animals (White et al., 1999).

APP is hypothesized to participate in the cellular copper efflux pathway (Treiber et al., 2004; Maynard et al., 2002; Hung, Bush, Cherny, 2010). Alternately, copper is involved in regulating APP expression (Cater et al., 2008; Bellingham et al., 2004; Armendariz, Gonzalez, Loguinov, Vulpe, 2004). To facilitate copper efflux, the metallochaperone Atox1 is responsible for the regulation of the trafficking of ATP7A (a copper-transporting, P-type ATPase) from the trans-Golgi network (TGN) to the plasma membrane (Petris et al., 1996; Hamza, Prohaska, Gitlin, 2003). Acevedo and colleagues (2011) have also shown that copper can promote redistribution of APP, through its exit from the Golgi to the cytoplasm and plasma membrane. However, copper does not play a

direct role in altering APP processing; therefore, copper is responsible for the regulation of APP localization (Acevedo et al., 2011).

As previously mentioned, there are two pathways of APP processing, the amyloidogenic and the non-amyloidogenic pathways. Intracellular copper deficiency can lead to increases in amyloid while enhancement of these intracellular copper levels promoted the non-amyloidogenic pathway through increased α-secretase cleavage (Cater et al., 2008). Copper increases α-secretase cleavage (Borchart et al., 1999). Cleavage of APP by β-secretase and γ-secretase leads to the amyloidogenic pathway and results in production of Aβ. An interaction between copper and the β-secretase, BACE1, has been found as copper plays a regulatory role in BACE1 expression by binding to it (Angeletti et al., 2005; Lin et al., 2008). This interaction between copper and BACE1 in important to the regulation of APP cleavage by β-secretase (Hung et al., 2010). Intracellular copper levels play an important role in APP processing. In the brains of TgCRND8 mice, ATP7A is over-expressed in activated microglia cells localized around β-amyloid. It is suggested that microglia sequester copper to provide neuroprotective effects in AD, as supported by the pro-inflammatory cytokine interferon-gamma increasing ATP7A expression and causing changes in copper homeostasis (Zheng et al., 2010).

Copper intake can lead to mental decline in normal individuals with diets high in saturated and trans fats (Morris et al., 2006) and in healthy older women (Lam et al., 2008). Salustri and colleagues (2010) reported that increased levels of free copper, not including bound copper, are a possible risk factor for cognitive decline that can be detected at an early stage in healthy individuals. Free copper is copper not bound to ceruloplasmin, and although may be bound to albumin or other small molecules, it is more available to meet cellular needs (Squitti et al., 2010). Sparks and Schreurs (2003) showed that in a population of cholesterol-fed rabbits, adding copper to the drinking water created memory deficits compared to high cholesterol animals not receiving copper supplementation. These results are relevant in AD patients with high cholesterol diets or those with late-onset APOE genetic markers; however, high cholesterol is not a hallmark of the CRND8 mouse mode or necessarily of early-onset familial AD pathology.

There are two disorders that exemplify inborn errors in copper metabolism, Menke's syndrome and Wilson's disease. Menke's syndrome is an inherited disorder of copper deficiency, occurring due to a mutation in the *ATP7 A* gene (Kaler, 1998; Brewer, 2000). Those with this disorder are unable to absorb adequate nutritional levels of copper from the gastrointestinal tract. As the nutritional needs are not met early in life, there is improper brain growth and motor neurodevelopment (Kaler, 1998). Treatment with copper histidine can allow for increased chance of survival in those with milder phenotypes (Kaler, 1998). Wilson's disease is an inherited disorder of abnormal copper accumulation and toxicity. Multiple mutations can be present in the *ATP7 B* gene, which causes an enzymatic deficit in the pathway of biliary excretion of excess copper (Brewer, 2000). Copper over accumulates in the brain and liver, resulting in neurologic and cognitive decline with liver disease (hepatitis and cirrhosis). Treatment can be effective if the patient is treated early with anticopper therapy (Brewer, 2000). These two copper based disorders are important because they show that both deficiency and overload of copper can lead to cognitive impairment.

Ceruloplasmin (Iron & Copper) in AD

Ceruloplasmin (CP) is a multicopper ferroxidase enzyme that is the major copper carring protein in the blood and plays a role in the metabolism of iron. CP exhibits a copper-dependent oxidase acivity, which is crucial to the oxidation of Fe^{2+} to Fe^{3+} (ferrous to ferric iron), therefore sufficient levels of CP may be required to prevent a dangerous build-up of Fe^{2+} (Reeves & DeMars, 2004; Yangisawaa et al., 2009). The oxidation of iron allows for its binding to transferrin, mobilizing iron through the body (c), allowing for efficient cellular iron release (Madsen & Gitlen, 2007). CP is synthesized in hepatocytes of the liver and is also one of the few proteins which have been established as playing a critical role in brain iron homeostasis (Madsen & Gitlen, 2007). CP is expressed in the glia and astrocytes of the brain, but not neocortical neurons (Klomp et al., 1996).

In AD patients, the concentration of CP appears to be normal (Castellani, Smith, Nunomura, Harris, Perry, 1999; Torsottir et al., 2010) or increased (Giometti, Argentiero, Sanson, Ongaro, Tavolato, 1988; Squitti et al., 2005; Lutsenko, Gupta, Burkhead, Zuzel, 2008), although with lower activity (Snaedal et al., 1998; Boll, Alcarez-Zubeldia, Montes, Rios, 2008). In AD and other progressive neurodegenerative conditions, CP activity declines during the course of the disease. Torsottir and colleagues have posed the question of whether the CP enzyme has the possibility of involvement in AD pathogenesis- either through a direct effect in the cascade leading to AD or an indirect effect resulting from increase dysfunction and increased oxidative stress (Torsottir et al., 2010).

The link between copper and iron in the function of CP makes it an important enzyme to examine in a study of metal overload. This link could also be why AD Tg animals who receive iron and copper supplementation together, may perform better than animals who only receive iron because of the compensatory mechanism served by CP when access to both metals is provided.

Trace Metals in the Liver

With AD there is an accumulation of Aβ plaques in and neurodegeneration of the brain, therefore it is an obvious choice to examine the changes in metal concentration of the brain. It may also be of interest to examine metal accumulation in other tissues in a mouse model of the disease. The liver is a necessary organ for survival and one important function is detoxification of chemicals and metals, as well as metabolizing drugs. The liver stores iron and copper as part of its normal function. However, overload of these metals is represented in diseases such as hemochromatosis (iron deposition in the liver) and Wilson's disease (copper build-up in the liver). So, it is interesting to determine whether excess concentrations of metals may end up accumulating in the liver in cases of dietary metal supplementation, especially in the case of Alzheimer's disease.

A unique property of copper is that the balance of copper is regulated by the liver (Squitti et al., 2007). Squitti et al. (2006) reported that AD patients may have abnormal copper incorporation into ceruloplasmin. In an examination of the possible link between copper dysfunction in AD and liver metabolism, they examined liver function in human AD patients. These patients, who had no additional pathological conditions, had higher

free (non-bound) copper than matched controls (Squitti et al., 2007). Also, a study of APP knockout mice showed copper levels were significantly increased in the cortex and liver compared to wild-type mice (White et al., 1999). White et al. (1999) concluded that APP expression lowers copper levels in the liver.

A study of ataxia-telangiectasia (A-T), a disease of chronic oxidative stress (OS), showed that increased iron accumulation in the brain is associated with hepatic changes (McDonald et al., 2011). Iron associated OS is linked with AT, so changes in the liver function due to iron loading in AT is relevant to dietary supplementation of iron in AD. By 4 weeks of age, AT-phenotype mice on iron developed iron loading in the liver (McDonald et al., 2011). Rats that were fed a dietary overload of iron in their food, had resulting increased levels of iron accumulated in the small extracellular foci of the liver and (iron storage-induced) DNA damage (Yang, Nair, Barbin, Bartsch, 2000). DNA changes were also seen in the livers of rats with iron overload and of patients suffering from primary hemochromatosis (Nair et al., 1998). APP knockout mice fed a normal diet had significantly higher levels of iron in the liver than age-matched controls, and knockout mice on a high-iron diet accumulated significantly more iron in the liver than those on a normal diet (Duce et al., 2010).

Behavioral Testing of Alzheimer's disease Transgenic Models

In human AD, large numbers of senile plaques and neurofibrillary tangles (NFTs) are found in the brain and are used to make a definitive, pathological, diagnosis of AD during postmortem autopsy. Senile plaques and NFTs are comprised of Aβ and APP (as discussed in the previous section *Interaction of Trace Metals and Alzheimer's disease*). Prior to postmortem diagnosis, a general diagnosis of dementia of the Alzheimer's type can be made based on symptoms such as cognitive deficits and memory loss.

It would be unethical to perform the type of research needed to understand disease pathology on human subjects, therefore, scientists use transgenic models. The mouse is considered to be a primary mammalian model system for genetic research due to the close genetic and phenotypic similarities to humans. The ease of genomic manipulation in the mouse model allows for genetically engineered mutants specifically designed to meet the needs for researching individual diseases (Spencer, 2002). So to study the effects of dietary metal supplementation, and resulting metal accumulation, on amyloid and AD mouse models must be used. Current genetic manipulations of different mouse models may result in the following pathological changes amyloid-beta plaques only, tau mediated tangles only, or a combination of both. For the proposed study, the examination of Aβ plaques will be the most beneficial to determine the effects of metal accumulation as these specific trace metals already play a specific role in the formation of senile plaques. The APP gene naturally carried by mice does not produce AD pathology, so transgenes are used to create various forms of hAPP (human amyloid precursor protein) mouse lines. These models result in both amyloid deposition and progressive memory loss which are pathological and behavioral hallmarks of AD.

One of the common transgene lines is the APP2576 mice which carries the Swedish mutation. The Swedish mutation is a missense mutation located before the amyloid-β peptide region of APP which causes an increased production of Aβ (Haass et

al., 1995). The Tg2576 mice (or transgenic APP2576 mice) have resulting cognitive deficits starting between 3 to 15 months (Hisao Ashe, 2001) which have been shown in spatial tasks such as the Morris water maze (MWM; Hisao et al., 1996) and T-maze (Chapman et al., 1999). This mouse model was previously tested in our lab, with resulting cognitive deficits at 18-21 months in the MWM (Railey, Groeber, Flinn, 2011) and NOR (novel object recognition is a visual recognition task; Groeber, 2009). APP_{SW} transgenic animals show sensorimotor and cognitive changes which are progressive (3 months to 9 months) and gender-dependent (King, et al., 1999). Notably these animals show impairment in the circular platform (Barnes Maze) and MWM retention & visible platform. Transgenic males showed increasing activity over controls in the open field and Y-maze tasks (King et al., 1999). Overall deficits appear to be most visible at 3 months of age with gender related differences more evident at 3 months; although this could be due to a natural decline in performance by controls at 9 months.

Specific to this proposed research, previous studies of enhanced metal water in our lab in the Tg2576 mouse model of AD, have shown that long-term dietary supplementation of zinc-enhanced water showed impairments in the spatial MWM task (Linkous et al., 2009) and those who received supplementation of zinc plus copper-enhanced water showed significantly less impairment (Railey et al., 2011). Enhancement with copper appears to show remediation of the deficits caused by dietary zinc-supplementation in AD-type transgenic animals. Tg2576 animals raised on iron-enhanced water showed similar deficits in the MWM to those raised on zinc-enhanced water (Railey et al., 2011). Tg2576 mice on zinc- and iron-enhanced water also showed impairments in the NOR task, with decreased novel object recognition as well as decreased sniffing time, compared to wild-type animals (Groeber, 2009).

A newer transgenic AD model, which progresses through the disease at a faster rate, is the TgCRND8 mouse model. It is a double mutant Swedish (KM670/671NL) plus Indiana (V717F) mouse which expresses the human APP 695 transgene (Chishti et al., 2001), also referred to as a Westaway mouse. The TgCRND8 mice begin to show deficits of reference memory at 11 weeks, correlating with the presence of amyloid at 3 months and the appearance of dense core plaques and neuritic pathology at 5 months of age (Chishti et al., 2001). Hyde et al. (2005) further classified age-progressive behavioral changes of CRND8 mice, showing changes tied to age-related neuropathology. There were no age differences in the Y-maze, with normal STM in the Tg mice. However, MWM impairments were present in early and late plaque Tg mice. While pre-plaque Tg mice showed normal MWM spatial learning compared with WT mice (Hyde et al., 2005). These findings correlate with data that Aβ40 and Aβ42 increase with age (Hyde et al., 2005).

In a study of leptin effects in 4-8 week old TgCRND8 mice, poor performance was exhibited by non-leptin treated TgCRND8 animals compared to WT animals in the NOR. Leptin is a hormone which controls feeding behavior, and in addition to its physiological effect, it is believed to improve memory and long-term potentiation (LTP) when directly injected into the hippocampus. Control TgCRND8 animals showed an average discrimination score of less than 0.2, well below the score of 0.5 required for "remembering" (Greco et al., 2010). Thus showing that standard TgCRND8 animals

show NOR deficits, but treatment with leptin was able to compensate for these disease modulated deficits. Linkous et al. (2009) conducted a study using both Tg2576 and TgCRND8 mice given dietary zinc supplementation. Both wild-type and transgenic mice, of both strains, showed impairment in spatial memory, as assessed through MWM. Transgenic animals given zinc supplementation showed the greatest impairments of all subjects, regardless of transgenic strain (Linkous et al., 2009).

Gender Differences and Alzheimer's disease

Women are at a higher risk for AD than men (Schmidt et al., 2008). For women, the risk of dementia and AD increases with age, especially in the oldest old group (90 years of age and older) (Yaffe et al., 2011). Women also seem to be more susceptible to cognitive deficits during the progression of AD, more so than male counterparts. Women who do not suffer from any cognitive impairment have better performance on semantic and verbal tasks (oral verbal fluency, written word fluency, naming, verbal learning) than men (Chapman et al., 2011). However, AD related cognitive impairments appear to reverse this advantage (McPherson, Black, Buckwalter, Cummings, 1999). AD-diagnosed women have greater deficits on tasks which require access to their personal knowledge base (i.e., semantic memory), as well as language ability and episodic verbal memory (McPherson et al., 1999). There does appear to be a gender difference in AD; one way to distinguish this difference is the larger gap in cognitive change from a healthy individual to an AD-diagnosis in women versus men (Chapman et al., 2011).

This difference may be due to post-menopausal hormone changes (i.e., estrogen and progesterone levels) (Turner, 2001; Baum, 2005). The current hypothesis on gender differences in AD includes estrogen deprivation as a fundamental factor. This is particularly important as menopausal changes in older women bring about loss of estrogen; the loss of which may increase susceptibility of the aging brain to neurodegeneration due to AD (Gandy & Duff, 2000).

More amyloid is measured in the brains of senescent female transgenic mice than the brains of older male mice (Callahan, Lipinski, Bian, Durham, Pack, Walker, 2001). When amyloid plaques are formed, the Aβ levels increase more significantly in females compared to same-age male transgenic animals (Hirata-Fukae et al., 2008). Impairments in behavioral tasks can be seen at different ages in male and female transgenic mice. APP_{SW} transgenic males showed a progressive impairment (from 3 months to 9 months) on the circular platform task, while transgenic females only showed impairments at 3 months (King et al., 1999). King et al. (1999) believe the differences in activity of transgenic males and transgenic females could be due to the interaction of Aβ and testosterone levels in the brain of male mice.

Estrogen, as an antioxidant, can protect against neuronal damage caused by Aβ accumulation (Goodman, Bruce, Cheng, Mattson, 1996; Wickelgren, 1997). It can also help with the maintenance of memory functions through interactions with acetylcholine in the hippocampus (Wickelgren, 1997). Consequently, a drop in estrogen levels in older, post-menopausal women could be the cause of the difference between the prevalence of AD in women and men. While production of ovarian hormones is slowed or stopped in older women, older men still produce testosterone and some of it is converted to estradiol

(Seeman, 1997). Estrogen replacement therapy (ERT) in women has been shown to delay AD (Paganini-Hill & Henderson, 1996).

The current growth of the number and age of the older adults living in the United States is unprecedented in our nation's history. Two factors- longer lives and aging baby boomers- will double the population of Americans ages 65 or older during the next twenty-five years. Life expectancy in the U.S. has increased from 47 years for an American born in 1900 to 77 years for those born in 2001 (CDC, 2003a) and baby boomers (those born between 1946 and 1964) will begin to reach age 65 in 2011. As mentioned, by 2030, the number of older Americans is expected to reach 71 million or an approximated 20 percent of the U.S. population (CDC, 2003b; Wan, Sengupta, Velkoff, DaBarrow, 2005). The increase in the number of older adults will expose us to a greater incidence of disease than has been experienced in the past. This is why it is important that diseases seen in the older adult population, like Alzheimer's disease, are studied to work towards potential therapies.

APPENDIX B: SPECIFIC AIMS AND HYPOTHESES

Aim One

The first aim of this study was to examine the effects of trace metals in the etiology of Alzheimer's disease. Specifically, we examined the effects of iron- and copper-enhanced water on hallmark pathological brain changes and cognitive learning and memory deficits, as assessed was through NOR and MWM.

Although iron is one of the most abundant metals in the body, an excess of ferrous iron in the brain can lead to neuronal degeneration further leading to an increase in Aβ plaque formation (Lovell et al., 1998). It has been suggested that decreased levels of copper in the brain could further increase Aβ production, bringing about a pathogenic cascade of events (Maynard et al., 2005). Furthermore, elevation of brain copper levels improved the survival of mice and resulted in the marked decrease in Aβ, supported by studies of dietary enhancement (Bayer et al., 2003) and mutant alleles (Phinney et al., 2003). It has been shown that copper rescues a zinc effect (Railey et al., 2010, 2011), but there has not yet been an examination of the effect copper would have on enhanced iron supplementation. A major aim of this study was the examination of whether enhanced copper could also remediate the iron effect. If so, this could suggest that copper remediation acts by increasing the non-amyloidogenic pathway and by reducing the amyloidogenic pathway and that obtaining a correct metals balance could be an important therapeutic factor for AD.

Hypothesis 1: Animals raised on iron-enhanced water have increased deficits in NOR and MWM relative to animals raised on lab tap water performing these tasks.

Hypothesis 2: Animals raised on copper-enhanced water have reduced deficits in NOR and MWM relative to animals raised on lab tap water performing these tasks.

Hypothesis 3: Animals raised on iron plus copper-enhanced water have reduced deficits in NOR and MWM relative to animals raised on iron-enhanced water, but significantly different from animals raised on lab tap water.

Hypothesis 1-3 Summary: Behavioral task performance (NOR, MWM) by animals on metal-enhanced water is as follows:

$$\text{iron} < \text{iron-plus-copper} \leq \text{lab water} < \text{copper}$$

Aim Two

Female gender is a risk factor for AD (Schmidt et al., 2008) and the risk increases with age (Yaffe et al., 2011). Cognitive decline even appears to be more severe for women when they are diagnosed with the disease (Chapman et al., 2011; McPherson et al., 1999).

> *Hypothesis 4:* Transgenic females have greater cognitive deficits than transgenic males, regardless of the water group on which they were raised.

Aim Three

A final aim of this study was to determine if dietary manipulation of these metals cause a change in the accumulation of Aβ and senile plaques in the brain. Congo red is a specialized histological analysis which was run to determine the level of plaque burden in the brain.

> *Hypothesis 5:* Amount of amyloid accumulation in animals raised on metal-enhanced water present as follows: iron ‹ iron-plus-copper ‹ lab water ‹ copper

APPENDIX C: METHODS, COMPLETE

Experimental Animals

Animal subjects in this experiment were transgenic (Tg) double mutant (Swedish: KM670/671NL plus Indiana: V717F, which express the human amyloid precursor protein transgene) mice, also referred to as Westaway mice. Wild-type animals were on the C3HxC57 background. Breeding pairs of these mice were obtained through a donation from the Center for Research in Neurodegenerative Diseases (CRND) at the University of Toronto. Breeding procedures are described later in the "*Breeding*" section. A power analysis was conducted using G*Power (Faul, Erdfelder, Lang, Buchner, 2007) to determine that a sample size of 12 animal was desired per group for appropriate power to be achieved. The power analysis was run assuming a medium effect size and an alpha level of $p<0.05$. As these mice have a high attrition rate (mortality rate of 25-40%) by 3 months of age (Chishti et al., 2001), larger group sizes were planned to protect for final power. Treatment groups consisted of lab tap water; iron enhanced water; copper enhanced water; and iron plus copper enhanced water, with wild-type and transgenic CRND8 mice randomly assigned to the treatment groups. Final numbers in treatment groups were as shown (Table 2). As there were a small number of original breeder pairs and high attrition rates, each group of offspring was relatively small. There were six total offspring groups which were each tested in independent rounds of NOR and MWM, but analyzed together due to these small numbers of offspring in each group.

Table 2. Groups of Tested Animals.
A Transgenic; B Wild-types. Fe = iron water; Cu = copper water; Fe plus Cu = iron plus copper water; Lab = lab tap water.

A

N = 11	Female	TgCRND8 + Fe	Male	N = 9	
N = 13	Female	TgCRND8 + Cu	Male	N = 8	
N = 10	Female	TgCRND8 + Fe plus Cu	Male	N = 8	
N = 9	Female	TgCRND8 + Lab	Male	N = 8	Total = 76 mice

B

N = 13	Female	Wt + Fe	Male	N = 11	
N = 9	Female	Wt + Cu	Male	N = 9	
N = 12	Female	Wt + Fe plus Cu	Male	N = 9	
N = 13	Female	Wt + Lab	Male	N = 13	Total = 80 mice

Animals were group housed, three to four per cage, with each cage containing two igloos with one wheel attachment and one Nyla bone. When fighting resulting in injury was seen in cages, the aggressive and/or injured animals were separated. All animals were handled daily, maintained on a diet of regular mouse-chow and provided access to food and water *ad libitum*. Consumption of water was measured, by cage, and recorded for later analysis. Mice were raised on non-enhanced lab tap water from birth through 5 weeks of age and then transitioned onto supplemented water groups until sacrifice. Behavioral testing occurred at approximately 5 months of age, when animals had consumed metal-supplemented water for approximately 4 months; total time of consuming metal-supplemented water was for approximately 5 months, including behavioral testing. Animals were tested using the novel object recognition (NOR) task and the Morris water maze (MWM) between five and six months of age. Animals were genotyped by Transnetyx for the confirmation of the presence or absence of the desired hAPP gene before weaning. CRND8 mice show brain pathology at 5 months (Chishti et al., 2001) similar to APP2576 mice at 12 months (Hisao Ashe, 2001). They also show similar physical signs of aging such as graying and thinning fur and changes in gait. These changes are also observed in Wt mice. 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.

Water Preparation

Water was prepared in the Biopsychology laboratory at George Mason University (GMU, Fairfax, VA). Iron-enhanced water, 10ppm $FeNO_3$, was prepared with the addition of an iron standard (Iron, %5HCl) to laboratory tap water. Copper-enhanced water, 0.2ppm $CuNO_3$, was prepared with the addition of a copper standard (Copper, %5 HNO3) to laboratory tap water. Iron- plus copper-enhanced water, 10ppm $FeNO_3$ with 0.2ppm $CuNO_3$, was prepared with the addition of an iron standard (Iron, %5HCl) and a copper standard (Copper, %5 HNO3) to laboratory tap water. Iron and copper metal standards were purchased from SPEX CertiPrep Group, Metuchen, NJ, USA. Laboratory tap water was obtained from the main water supply of the laboratory in David King Hall (GMU). The pH of the enhanced metal water was tested after preparation to ensure proper pH for dietary consumption.

Breeding

Breeders of 6-8 weeks of age were obtained from CRND at the University of Toronto (referred to as Breeders 1). The Westaway mouse (TgCRND8), expresses human APP with the Swedish and Indiana mutations. These mutations have both been linked to familial AD. This mouse model develops amyloid deposits by 3 months of age with a significantly higher ratio of Aβ42 than Aβ40. Animals were allowed to acclimate to the facility for a period of 2 weeks prior to breeding; females were group housed and males were individually housed. Donated breeders were used to create a second line of breeders (referred to as Breeders 2). There were a total of 5 rounds of breeding to obtain the desired number of animals for behavioral testing. Mice were harem bred; one male

was paired with three to four female mice for 5-8 days, to ensure they were paired together during an estrous cycle. The male was introduced to a female when she was 8 weeks or older. After this time period (pairing), males were immediately removed and returned to their individual cages. Although moved to a new cage, females were kept together to reduce stress until approximately a week before the expected birth of the pups when each animal was moved to separate cages with new bedding where they would remain until pup weaning. Mice were housed in a climate controlled 22°C ± 2°C environment with a 12:12 light/dark cycle and *ad libitum* access to food and water.

Experiment 1: Novel Object Recognition

The hippocampus is one region of the brain in which amyloid plaques first appear and is an important area of the brain for learning and memory. Clark, Zola, and Squire (2000) reported that lesions of the hippocampus do impair performance on tasks of visual recognition memory and also that the hippocampus is critical for spatial memory. The NOR task can be used as an assessment for changes in learning and memory deficits, specifically deficits associated with recognition memory. NOR is one of the few tests available for assessing declarative memory (or more specifically episodic memory) in animals and is a standard task used with rats, non-transgenic mice, other mammals, and very young children, but not yet in transgenic mice (Mumby, 2001). In this task, wild-type mice will choose a novel, unfamiliar object showing the capability to distinguish between objects with which they have or have not had previous experience. A 'choice' is made between two objects through sniffing behavior. As an animal approaches, and sniffs an object, that animal is showing interest in that particular object. It would be expected that a wild-type mouse would show preference of a novel object by spending more time sniffing on the novel object (no prior exposure), over the familiar object (prior exposure). This task does not depend on the motivation to seek food after food deprivation or to avoid aversive conditions such as to escape from water or bright light.

The novel object recognition (NOR) task was performed using animals at 5 months of age. The NOR box was a blue four-sided Plexiglas square box, 18 inches by 18 inches and 9.5 inches in height (Clever Sys., Inc., Reston, VA). The animals were black and brown mice with brown eyes, so the blue box was chosen as a neutral background to create the greatest contrast for the tracking computer system. Velcro was used to secure objects to the bottom of the NOR box. The objects used in this experiment were a small light bulb (upside down), a miniature trophy, a Lego structure, a baby block with an Easter egg glued on top, and a small kaleidoscope glued on top of a baby food jar.

The experimental room was dimly-lit with lights located on the ground facing the wall and away from the NOR box. A light meter was used to determine that all portions of the box are equally lit and that there are no shadows in the box. There was a noiseless fan operating near the box to help prevent the build-up of scents in the room. The door to the room was closed during the experiment to prevent extraneous light and noise from entering. A video camera attached to the ceiling was positioned over the NOR box to provide video for the CleverSys Object Scan Behavior Analyzing system (TopScan, Clever Sys., Inc.) which was used to track sniffing behavior. Sniffing behavior was measured through nose sniffing, when the animal points its nose directly toward the

object. Prior to the start of any testing day, animals were placed in transport cages and taken to a room near the testing room and allowed to habituate for a 10 minutes.

Habituation: The animals first had 5 days of habituation during which time they were placed in the NOR box and allowed to explore with varying paradigms each day (i.e., differing time spent in the box, object presence/absence). During the first day of habituation, animals were placed in the box with their cage mates or alone, if they had no cage mates, with no objects for a 7 minute duration. During days 2, 3, and 5 of habituation, the animals were placed in the box individually for a 6 minute duration, with no objects. On day 4 of habituation, the animals were placed in the box individually, for a 6 minute duration, with their standard object in the box. Varying durations of time spent in the box during habituation will be used to prevent the animal from habituating to a specific time duration spent in the testing box. Following habituation, there was 3 days (4 trials) of testing for novel object discrimination. During each trial there were 2 objects placed in the box and the animals was in the box for a 5 minute duration.

Object Recognition: The first day post-habituation, each animal was placed in the box for the first time with two of the same object; this object became the animals' standard (object). The standard was present in all other trials for the animal and was tested against a novel object in each subsequent trial. Following the initial exposure trial, the animal went through four more trials after delays of 15 minutes, 1 hour, 24 hours, and 48 hours. Fifteen minutes after the initial trial, the animal was placed into the box again, but this time one object was the standard and the second object was a novel object they had never seen before. Between trials on the first day, the (initial, 15 minutes, and 1 hour) the animal was placed back in its testing cage in the room where it habituated not back in the colony room. One hour later, the animal was again placed in the box with its standard and a second novel object. Twenty-four hours later, the animal was placed in the box with its standard and a third novel object. Forty-eight hours later, the animal was placed in the box with its standard and a fourth novel object.

Table 3. Schedule of Habituation Days

Day	# of Animals	Objects	Time in Box
1	All cage mates	None	7 minutes
2	Individual	None	6 minutes
3	Individual	None	6 minutes
4	Individual	One Standard	6 minutes
5	Individual	None	6 minutes

Table 4. Schedule of Object Recognition Days

Day	Testing Interval	Objects	Time in Box
1	Initial	Standard / Standard	5 minutes
1	15 minutes	Standard / Novel 1	5 minutes
1	1 hour	Standard / Novel 2	5 minutes
2	24 hours	Standard / Novel 3	5 minutes
3	48 hours	Standard / Novel 4	5 minutes

Counterbalancing was planned for water group, genetic type, object, and object location to ensure no confound due to these extraneous variables. For each animal, the standard object remained at one location during all trials and each novel remained in the opposite location. However, this was rotated between mice so that some have the standard presented on the left while others had the standard presented on the right.

Experiment 2: Morris Water Maze

The Morris water maze task (MWM) is designed to assess spatial (reference) memory function (Morris, 1984; D'Hooge & De Deyn, 2001). Animals must learn the location of a hidden escape platform relative to visual cues situated around the pool area. It is widely accepted that spatial memory is largely a function of the hippocampus. The hippocampus is a region that is damaged early on in AD and spatial disorientation and getting lost in familiar areas are often early signs of the disease. As MWM is considered to be a relatively stressful behavior task, it was conducted after NOR testing to reduce the risk of negative effects due to stress on learning and memory.

A Coulbourn Instruments WaterMaze (Allentown, PA) software and computerized tracking system was used for recording and analysis of behavior. The pool was a four-foot diameter pool, and was surrounded on all sides by white curtains, each side with a distinctive cue to serve as external maze reference points. The cues consisted of black cutout designs of various shapes and sizes on the four sides. The transparent Plexiglas platform was submerged 1cm beneath the water surface; on "probe trials" the platform was completely submerged. The pool was conceptually divided into four quadrants (NE, SE, NW, or SW) for the purpose of design and data analysis. The animals were placed in the pool at a different starting quadrant for each trial and the starting quadrant order was changed for each day of testing. The water was dyed with white, non-toxic tempera paint to help disguise the location of the submerged platform. Testing took place over a period of eight days.

Behavioral measures of interest were latency (the length of time required to find the platform from trial start), the number of platform position crossings on probe trials, the percentage of time spent in the target quadrant on probe trials, and thigmotaxia. Thigmotaxia is considered a measure of anxiety, and it defined as percent of time spent swimming the outermost 10% of the pool closest to the edge/wall. If the animals are highly anxious and spend a majority of the sixty second trial only swimming the outer edge of the pool, they cannot find the platform or effectively learn the task. It is

important to determine if an animal's poor performance on the task truly represents a memory deficit, or if it is more likely attributable to a failure to effectively search the pool area due to anxiety.

Atlantis Platform: On days one through seven the platform remained stationary in a predetermined quadrant location each day, 1cm below the surface of the water. Each animal had three, 60 second trials (A, B, C) per day for seven days, with a 45 second inter-trial interval. On every sixth trial (C trials on days 2, 4, 6), the mice were given a probe trial. On Day 7, animals were given a single 24 hour probe trial. During the probe trial the platform was made unavailable while the animal swam for 60 seconds; and the amount of time spent searching the target quadrant (quadrant which previously contained the platform) and the number of crossings over the submerged platform was measured. The platform was raised after one minute, and if the animal had not yet found the platform they were gently guided to it and given 15 seconds on the platform to observe the surroundings. As the location of the stationary platform must be recalled across days, this is considered a measure of spatial reference memory.

Visible Platform: Following the final probe trial on Day 7, the final day of testing (Day 8) consisted of two visible platform trials during which time the platform was elevated above the water level (1cm) and clearly identified with a marker (a large wooden dowel adorned with alternate stripes of black and white tape glued to it) to explicitly indicate its location. The platform was located on the opposite quadrant of the pool than it was located during Atlantis platform trials. These trials served to identify and eliminate any animals with sensory or motor deficits in order to prevent confounds due to an inability to perform the task rather than a true deficit in learning and memory. During visual platform, mice were given two 60 second trials, 45 seconds apart.

Table 5. Schedule of Morris Water Maze Days

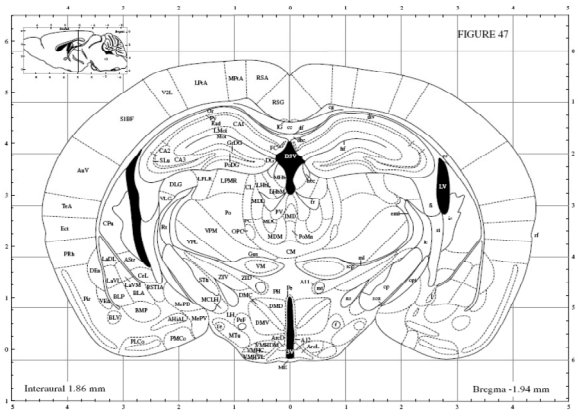
Day	# of trials	Platform Location	Length of trials	Inter-trial interval
1	A	Stationary (submerged 1cm)	60 seconds	45 seconds
	B	Stationary (submerged 1cm)	60 seconds	
	C	Stationary (submerged 1cm)	60 seconds	
2	A	Stationary (submerged 1cm)	60 seconds	45 seconds
	B	Stationary (submerged 1cm)	60 seconds	
	Probe 1	Probe (Atlantis, platform unavailable)	60 seconds	
3	A	Stationary (submerged 1cm)	60 seconds	45 seconds
	B	Stationary (submerged 1cm)	60 seconds	
	C	Stationary (submerged 1cm)	60 seconds	
4	A	Stationary (submerged 1cm)	60 seconds	45 seconds
	B	Stationary (submerged 1cm)	60 seconds	
	Probe 2	Probe (Atlantis, platform unavailable)	60 seconds	

5	A B C	Stationary (submerged 1cm) Stationary (submerged 1cm) Stationary (submerged 1cm)	60 seconds 60 seconds 60 seconds	45 seconds
6	A B Probe 3	Stationary (submerged 1cm) Stationary (submerged 1cm) Probe (Atlantis, platform unavailable)	60 seconds 60 seconds 60 seconds	45 seconds
7	Probe 24 hr	Probe (Atlantis, platform unavailable)	60 seconds	45 seconds
8	V 1 V 2	Visible, (above 1cm) Visible, (above 1cm)	60 seconds 60 seconds	45 seconds

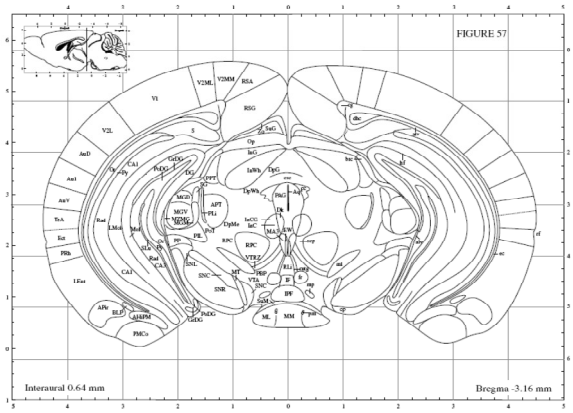
Histological Analysis

After behavioral testing was completed, mice were sacrificed for the use of brain and liver in histological analysis. Sacrifice and extraction occurred in a separate room from the home colony room. Mice were anesthetized with carbon dioxide (CO2; in cylinder) using an optimal flow rate which displaced at least 20% of animal chamber volume per minute. When the animal was unconscious, as indicated by no response to tail pinch, they were decapitated with a guillotine designed for rodent decapitation and brains and livers were extracted immediately. The brain and liver were immediately extracted. The brains were flash-frozen in dry ice, then stored in a -80°C freezer for longer term storage. Livers were fixed in 10% neutral buffered formalin (NBF) for future analysis.

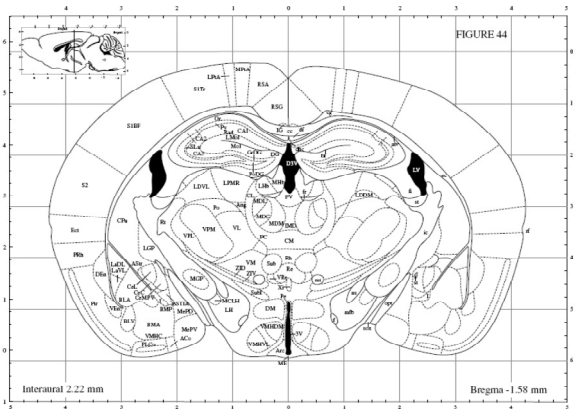
The left hemisphere of each brain was sectioned with a Leica CM3050 S cryostat in coronal sections ranging from 5 μm to 100 μm through the hippocampus, surrounding cortex, and basal ganglia. Depending on the planned use of each section, the section was placed on either a normal glass slide or a zinc-free Silica slide for the following discussed histological analyses. The Paxinos and Franklin (2001) atlas was used for consistency of region of interest (ROI) location during sectioning. The five predetermined ROIs are as follows: ventral hippocampus (vHC), dorsal hippocampus (dHC), anterior hippocampus (aHC), basal ganglia 1 (BG1), and basal ganglia 2 (BG2) (Figure 11). A Congo red stain procedure was the primary histological analysis for the assessment of plaque load in the left hemisphere of the brain. Additional sections of the left hemisphere were taken for future assessment with Perls' Prussian blue, Rhodanine, and Synchrotron X-ray Fluorescence (SXRF; brain only). The right hemisphere was preserved for analysis of amyloid (presence of and soluble vs. insoluble) and ceruloplasmin (CP) at a later date. Livers were preserved, fixed in 10% neutral buffered formalin (NBF) and paraffin embedded, for future histological analysis of iron load (Perl's Prussian blue) and copper load (rhodanine).



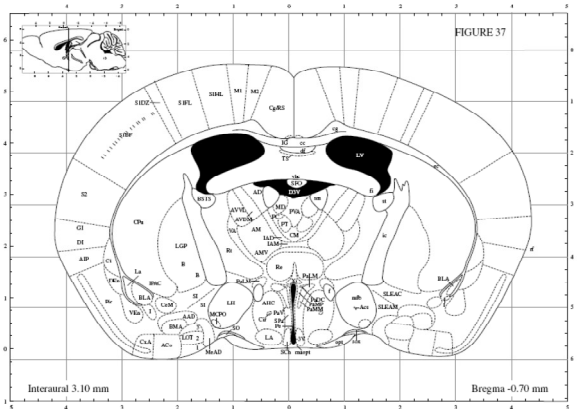
(a) ROI 1: vHC



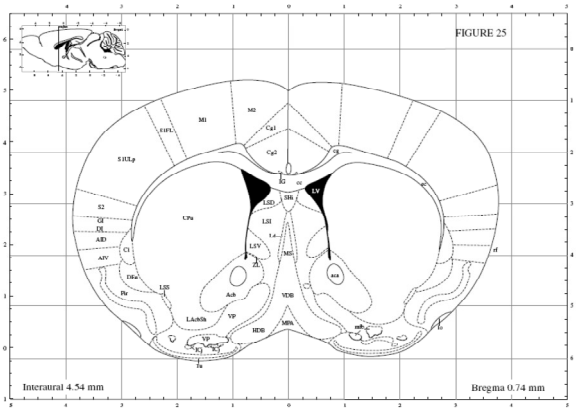
(b) ROI 2: dHC



(c) ROI 3: aHC



(d) ROI 4: BG1



(e) ROI 5: BG2

Figure 11. Regions of Interest (ROI 1-5) used for sectioning guidance.
Taken from Paxinos and Franklin (2001)

Congo Red

To determine if there are amyloid deposits and the amount of plaques in the tissue sections, Congo red stain was used. The protocol for Congo red staining is Putchler's Modification (convention method) from WebPath: Internet Pathology Laboratory (Florida State University College of Medicine, 2007).

1. Mayer's Hematoxylin for 10 minutes.
2. Wash in tap water until blue.
3. Working sodium chloride solution, room temperature, 20 minutes.
4. Place directly in working Congo red solution for 1 hour.
5. Dehydrate rapidly in absolute alcohol, 10 dips, 3 changes.
6. Clear in xylene
7. Coverslip with Permount.

After staining, amyloid stains red to pink and nuclei should stain blue. Stained sections were viewed with an Olympus BX51 fluorescent/polarizing microscope. Under the polarizing lens of the microscope, the stained amyloid birefringes an apple green color (Puchtler, Sweat, & Levine, 1962). ImageJ, a public domain, Java-based image processing program developed at the NIH (National Institutes of Health, 2013) was used to quantify images of the slices/amyloid. Image J can display, edit, analyze, process, and save, microscope images as well as has the ability to calculate area and pixel value statistics of user-defined selections, all properties which are necessary for the analysis of uniquely shaped and defined amyloid plaques.

Perls' Prussian Blue

To determine the amount of ferric iron (Fe^{3+}) in the tissue, Prussian blue stain was used. The protocol for Prussian blue staining is Mallory's Method from WebPath: Internet Pathology Laboratory (Florida State University College of Medicine, 2007).

1. Prussian blue working solution, room temperature, 30 minutes.
2. Rinse in deionized/distilled water, at least 1 minute.
3. Nuclear-fast red, 5 minutes.
4. Rinse in deionized/distilled water, at least 1 minute.
5. Dip slides in 95% ethanol.
6. Dip slides in 100% ethanol, two changes.
7. Clear in xylene.
8. Coverslip with Permount.

After staining, the ferric iron deposits (hemosiderin) appear bright blue as a reaction to the acidic ferrocyanide of the stain. The nuclei of cells (and hemofuschin) appear red and the background tissue appears pink due to the nuclear-fast red background stain used. Stained sections were viewed with an Olympus BX51 fluorescent/polarizing microscope and analyzed with ImageJ as discussed for the Congo red stain above.

Rhodanine

To determine the amount of copper deposits in the tissue, rhodanine stain was used. The protocol for rhodanine staining (a microwave method) is from *Histotechnology: A Self-Instructional Text* (Carlson, 1997).

1. Place slides in plastic Coplin jar of Working rhodanine solution (50 mL), the cap loosely applied
2. Microwave, power level 1 (60W), 5 minutes.
3. Dip the slides up and down, several times.
4. Allow the slides to remain in the hot solution, 3 minutes.
5. Microwave, power level 1, 90 seconds.
6. Allow the slides to remain in the hot solution, 15 minutes.
7. Wash with distilled water, 6 changes.
8. Diluted Mayer Hematoxylin, 10 minutes.
9. Rinse in distilled water, 2 changes.
10. Blue nuclei with 0.5% sodium borate, 5 seconds.
11. Rinse with distilled water, 4 changes.
12. Mount with an aqueous mounting medium.

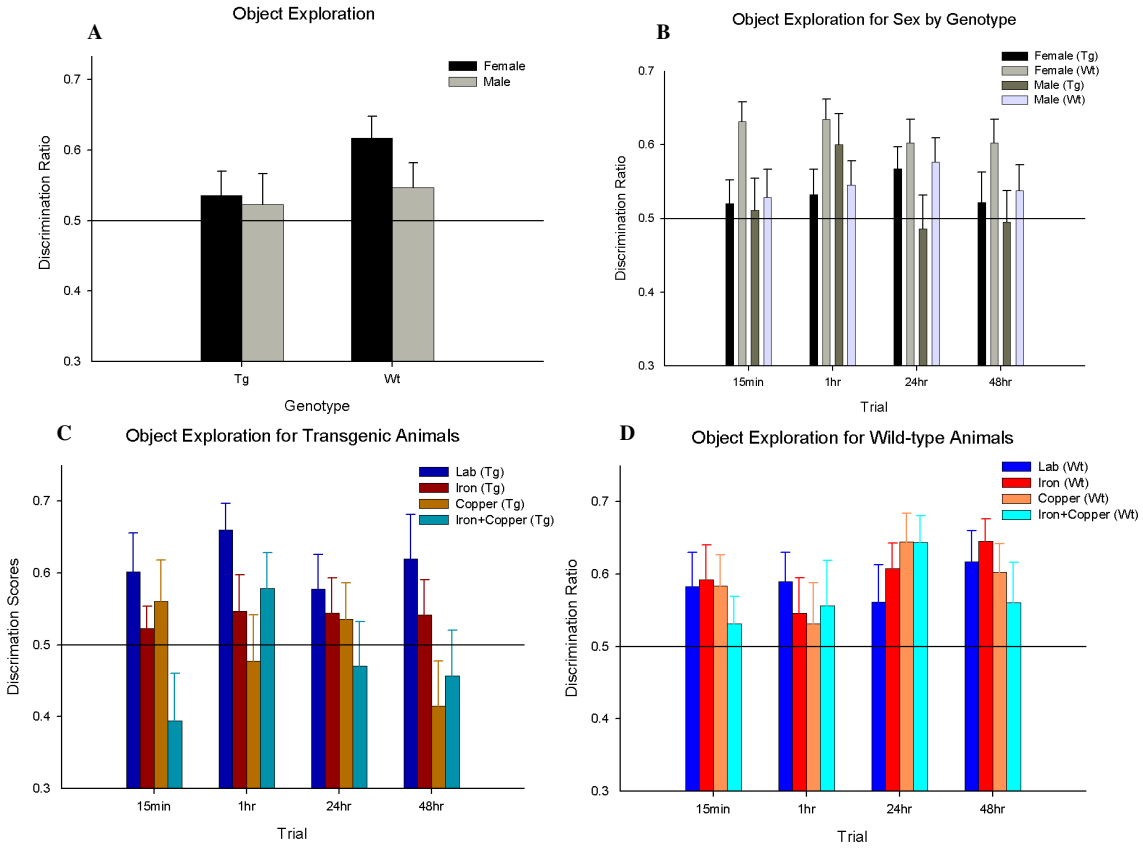
After staining, copper deposits appear as bright red to rust-red intra-cytoplasmic granules while the nuclei of cells appear blue. Stained sections were viewed with an Olympus BX51 fluorescent/polarizing microscope and analyzed with ImageJ as discussed for the other stains above.

Synchrotron X-ray Fluorescence (SXRF)

Additional sections were taken for further analysis at Brookhaven National Laboratory (BNL) to measure metal content in the brains of the transgenic animals. Coronal sections at 40 μm were mounted on zinc-free Silica slides. Samples were run at the National Synchrotron Light Source (NSLS) on the X27A beamline. The research program for X27A is "x-ray fluorescence (spectro)microscopy with emphasis on applications in the life, geo, and environmental sciences". Beam time was donated and paid for as a General User Program Grant through the Department of Energy (DOE).

APPENDIX D: COMPLETE RESULT FIGURES

Experiment 1: Novel Object Recognition



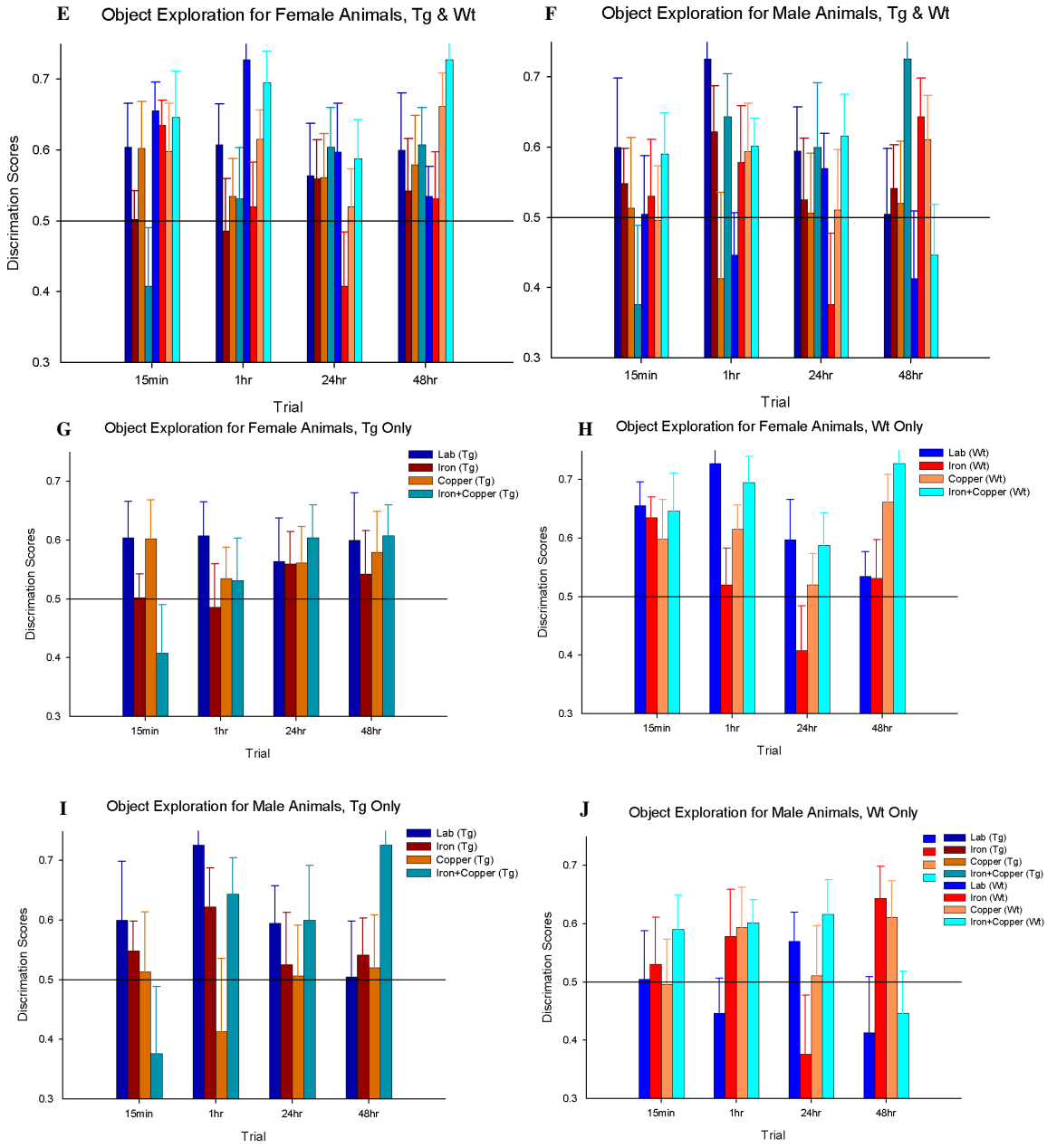


Figure 12. Object Exploration

Females showed better overall object recognition than males ($p<0.05$). There was no significant difference between Tg male and females, but Wt females did show more object recognition that Wt males ($p=0.018$). (Discrimination Ratio = novel object sniffing / (novel sniffing + standard sniffing)). **A.** By genotype and Sex; **B.** Sex and Genotype across Trials; **C.** By Water Groups for Tg; **D.** By Water Groups for Wt; **E.** By Water Groups for Females; **F.** By Water Groups for Males; **G.** By Water Groups for Female Tg; **H.** By Water Groups for Female Wt; **I.** By Water Groups for Male Tg; **J.** By Water Groups for Male Wt.

Experiment 2: Morris Water Maze

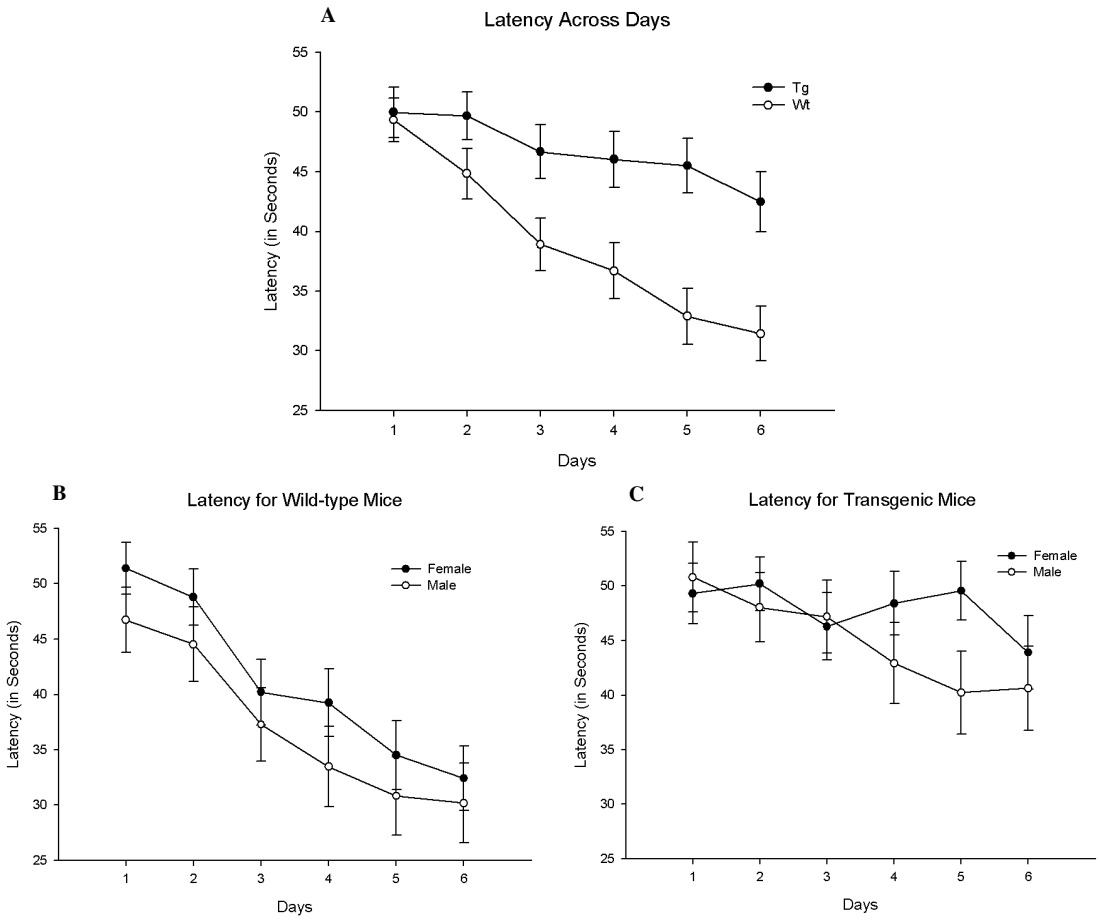
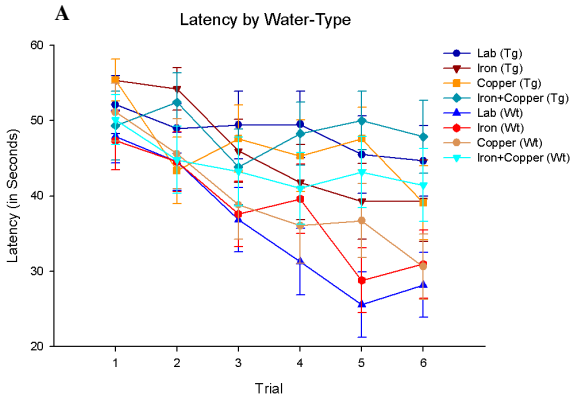
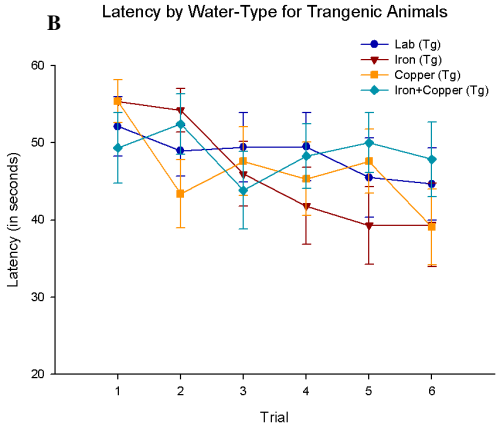
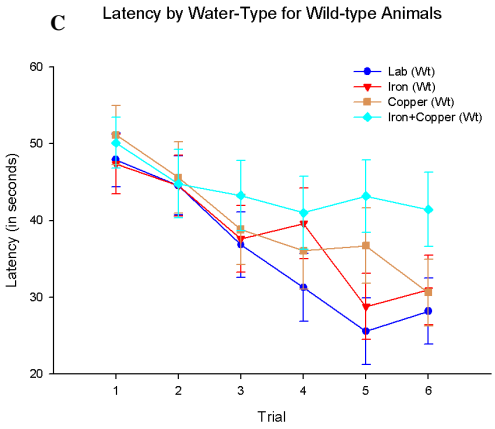
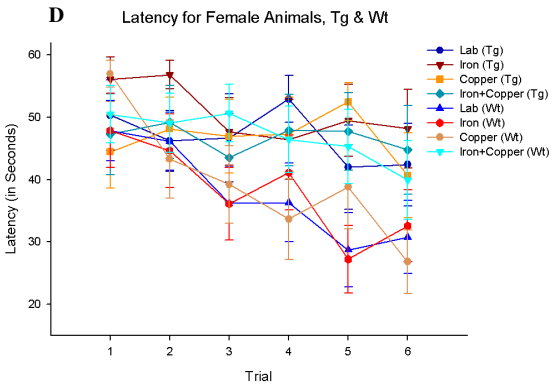


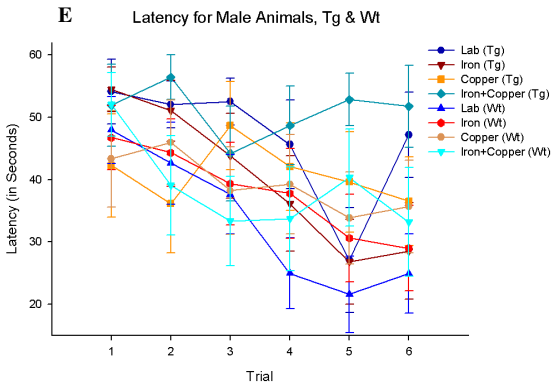
Figure 13. MWM Latency across Days 1-6, by Genotype and by Sex for Tg & Wt mice.
A. Tg mice had significantly longer escape latencies than Wt mice across all days of testing ($p<0.001$). **B.,C.** There is a significant difference in escape latency between the male and female animals, regardless of genotype ($p<0.05$).


A
Latency by Water-Type
Latency (in Seconds)
Trial
Lab (Tg)
Iron (Tg)
Copper (Tg)
Iron+Copper (Tg)
Lab (Wt)
Iron (Wt)
Copper (Wt)
Iron+Copper (Wt)


B
Latency by Water-Type for Trangenic Animals
Latency (in seconds)
Trial
Lab (Tg)
Iron (Tg)
Copper (Tg)
Iron+Copper (Tg)


C
Latency by Water-Type for Wild-type Animals
Latency (in seconds)
Trial
Lab (Wt)
Iron (Wt)
Copper (Wt)
Iron+Copper (Wt)


D
Latency for Female Animals, Tg & Wt
Latency (in Seconds)
Trial
Lab (Tg)
Iron (Tg)
Copper (Tg)
Iron+Copper (Tg)
Lab (Wt)
Iron (Wt)
Copper (Wt)
Iron+Copper (Wt)


E
Latency for Male Animals, Tg & Wt
Latency (in Seconds)
Trial
Lab (Tg)
Iron (Tg)
Copper (Tg)
Iron+Copper (Tg)
Lab (Wt)
Iron (Wt)
Copper (Wt)
Iron+Copper (Wt)

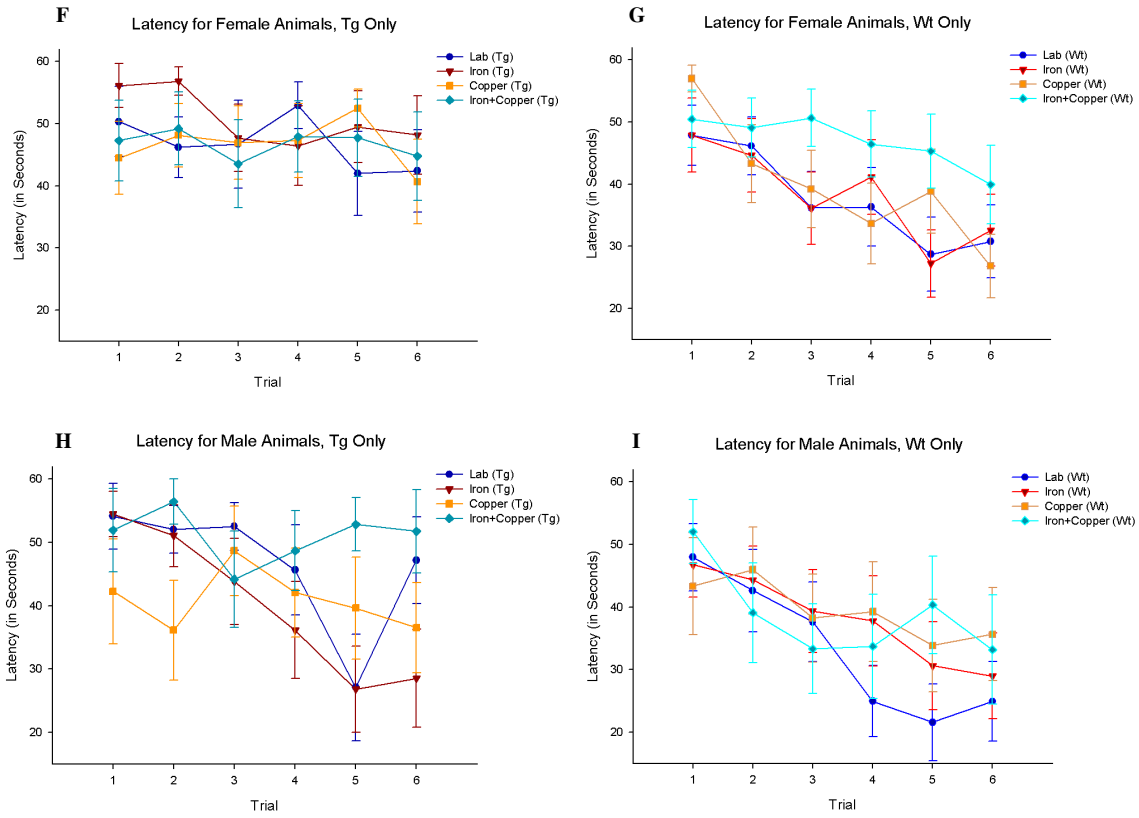


Figure 14. Latency across MWM Days 1-6, by Water-type and Sex
There were no significant effects of water group, but Tg mice had longer escape latency times than **A.** By Water Group; **B.** By Water Groups for Tg; **C.** By Water Groups for Wt; **D.** By Water Groups for Females; **E.** By Water Groups for Males; **F.** By Water Groups for Female Tg; **G.** By Water Groups for Female Wt; **H.** By Water Groups for Male Tg; **I.** By Water Groups for Male Wt.

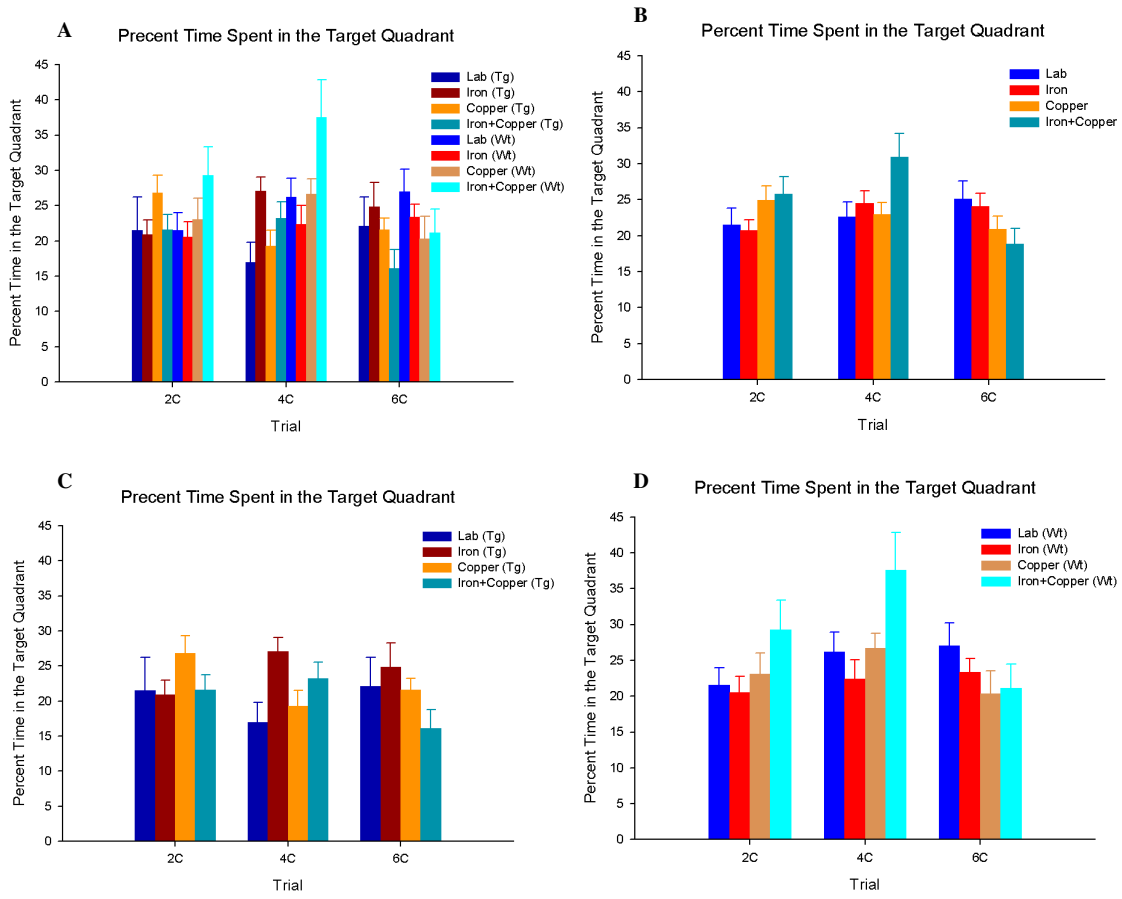


Figure 15. Percent Time in Target Quadrant during MWM Probe Trial Days 2,4,6.
Tg animals spent significantly less time in the platform quadrant than Wt mice ($p<0.05$), but there were no significant effects of sex or watertype. **A.** By Water Groups; **B.** By Water Groups, Collapsed for Genotype; **C.** By Water Groups for Tg; **D.** By Water Groups for Wt.

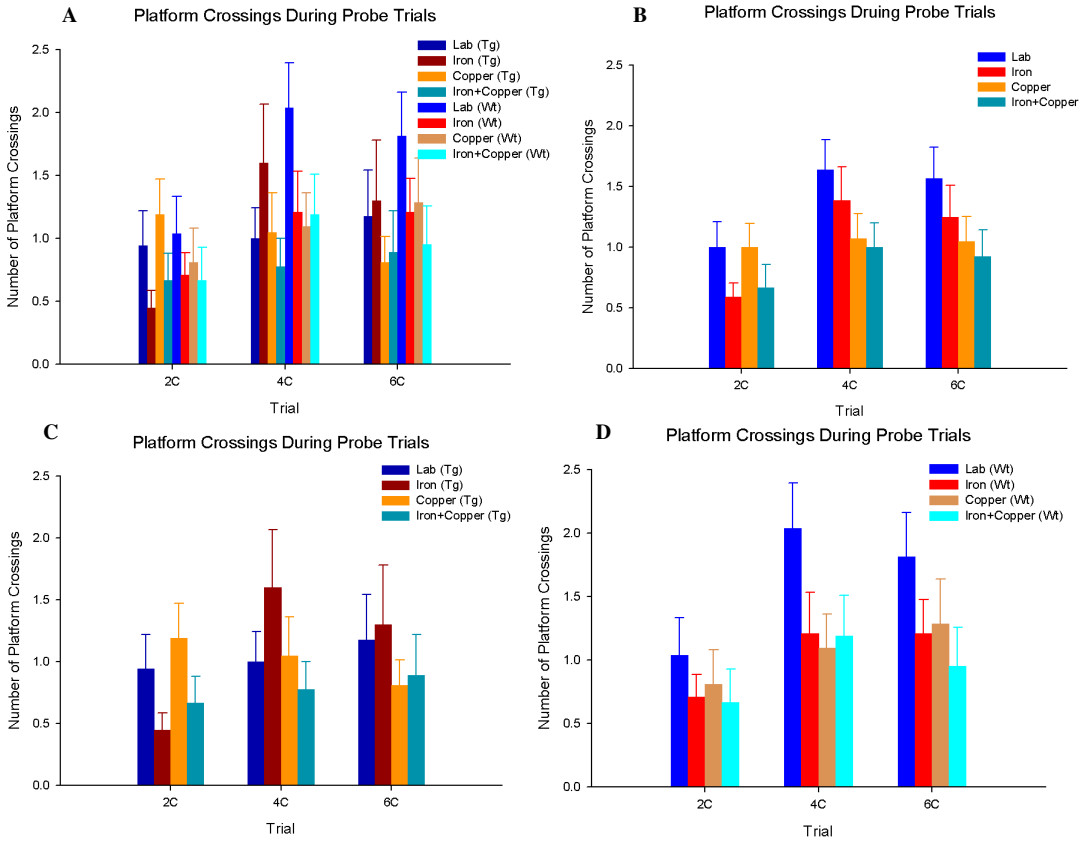


Figure 16. Number of Platform Crossings in Target Quadrant During MWM Probe Trials Days 2,4,6. There was no significant effect of genotype, sex, or water group. **A.** By Water Groups; **B.** By Water Groups, Collapsed for Genotype; **C.** By Water Groups for Tg; **D.** By Water Groups for Wt.

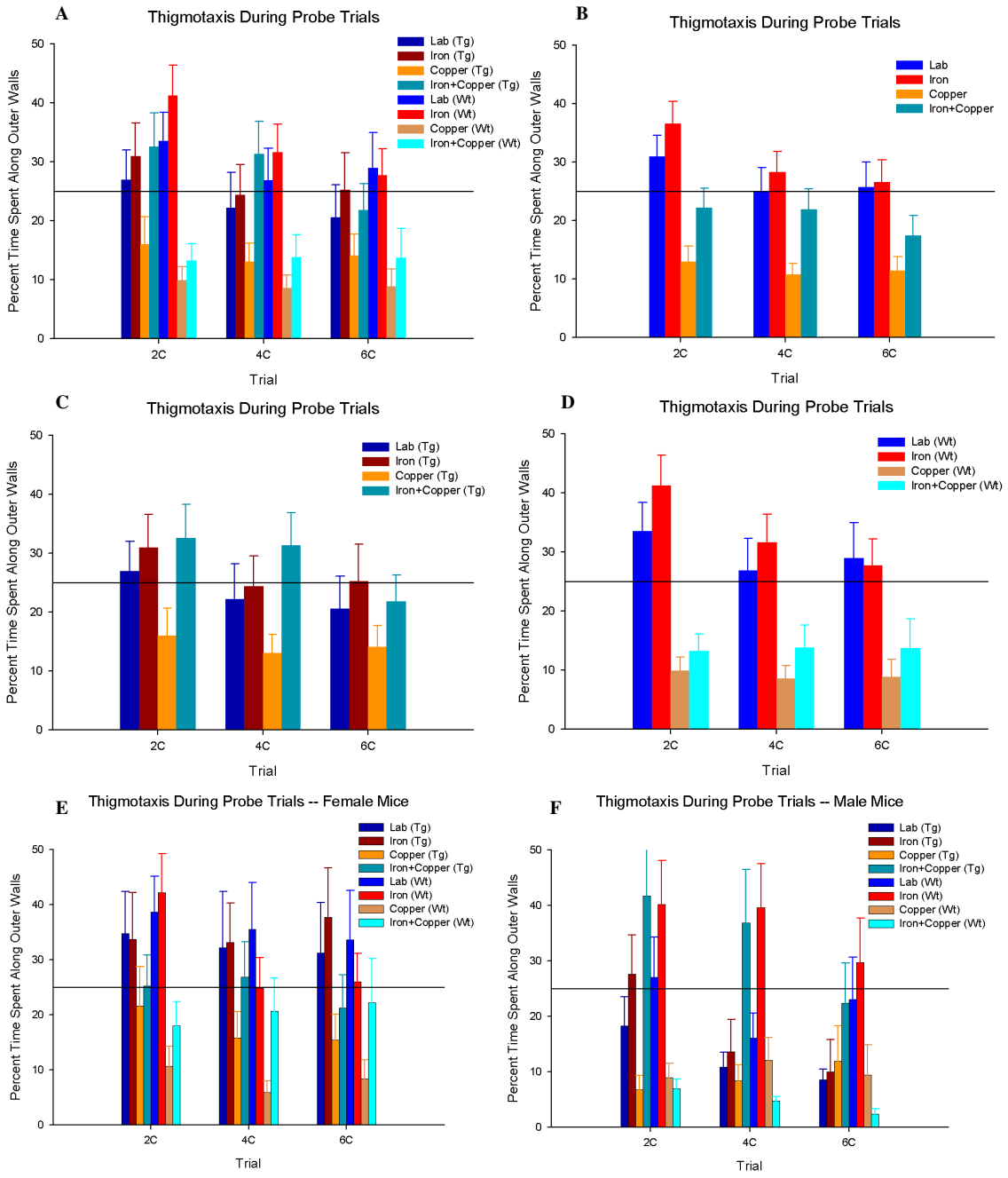


Figure 17. Thigmotaxia during MWM probe trials Days 2,4,6.
Mice spent significantly less time at the edge of the pool across probe trials ($p \leq 0.001$). Females ($p \leq 0.01$) and lab and iron-treated ($p \leq 0.001$) animals spent significantly more time at the edge of the pool than males and copper-treated animals, respectively. **A.** By Water Groups; **B.** By Water Groups, Collapsed for Genotype; **C.** By Water Groups for Tg; **D.** By Water Groups for Wt; **E.** By Water Groups for Female; **F.** By Water Groups for Male.

Histological Analysis

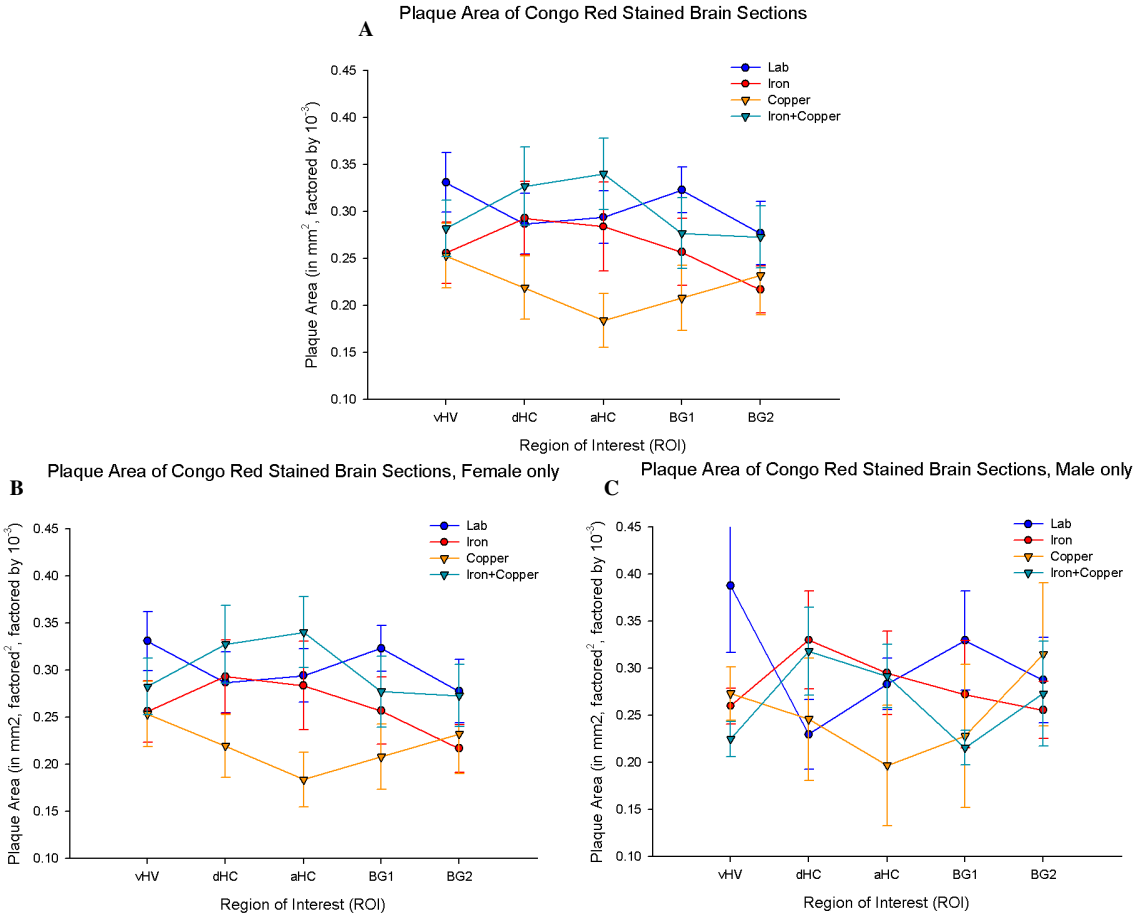


Figure 18. Plaque load levels.
There was no significant difference for water groups, although LW animals had significantly more plaques than Cu animals ($p<0.05$). Water deficits appear to be largely due to females as they did show a trend for significance across water groups ($p<0.1$) and plaque area across ROI ($p<0.1$). **A.** By Water Groups; **B.** By Water Groups for Females; **C.** By Water Groups for Males.

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