Neurobehavioral and neurophysiological effects produced by enhanced consumption of zinc, zinc plus copper, and iron in APP2576 mice as assessed through novel object recognition and histopathology

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

By

Caitlin M. Groeber
Bachelors of Science
Virginia Polytechnic Institute and State University, 2005

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

Summer Semester 2009
George Mason University
Fairfax, VA

DEDICATION

This thesis is dedicated to my mother, Ginger Groeber, who is eternally supportive of every decision made by her daughters and is a constant sounding board for my ideas.

To my sister, Emily Groeber, who is always there for me.

To my partner, Matthew Travis, who has remained by my side through the trials of an extended post-undergraduate education in the sciences.

To my grandmother, Virginia Coleman, who is the role model of vitality in old age and an inspiration for this research.

ACKNOWLEDGEMENTS

Thank you to Dr. Jane Flinn, Dr. Linda Chrosniak, and Dr. Patrick McKnight for remaining my committee members through this extended thesis project. Thank you for your guidance and continued support through out the inception, execution, and evaluation of this project.

Thank you to additional members of the graduate program without who the completion of this project would not have been possible, James Thompson, Erinn Gideons, Andrew Burns, Teresa Micheli, Katherine Cano, and Angie Railey.

Thank you to all of the others belonging to the George Mason University community who have been a part of this experience.

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ABSTRACT

NEUROBEHAVIORAL AND NEUROPHYSIOLOGICAL EFFECTS PRODUCED BY ENHANCED CONSUMPTION OF ZINC, ZINC PLUS COPPER, AND IRON IN APP2576 MICE AS ASSESSED THROUGH NOVEL OBJECT RECOGNITION AND HISTOPATHOLOGY.

Caitlin M. Groeber, MA

George Mason University, 2009

Thesis Director: Dr. Jane M. Flinn

This study examined the effect of drinking water enhancement with specific metal ions, which are commonly found in the brain, and the impact on an animal model of Alzheimer's disease (AD). Mice with the amyloid precursor protein (APP) Tg2576 mutation were used to examine the effect of metal iron overload on brain pathology and behavioral outcomes. Transgenic (Tg) and wild-type (wt) mice were placed into one of the following water groups zinc-enhanced water (10ppm $ZnCO_3$), zinc plus copper-enhanced water (10ppm $ZnCO_3$, 2ppm Cu), iron enhanced water (10ppm $FeNO_3$), or lab tap water. Two groups were tested, group 1 examined initial prenatal exposure to iron and group 2 examined initial exposure to all metals at adolescence (or three months of age). At twelve months animals were tested in the novel object recognition (NOR) apparatus, a behavioral assessment of recognition memory. After testing, animals were

sacrificed and histological brain analyses were conducted on transgenic animals to determine differential changes in plaque pathology by Congo red staining.

The results of this study showed that the NOR task has the potential to be used as a standard recognition (memory) deficit task for transgenic mice. Transgenic animals consistently sniffed for a shorter duration on the novel object than the wild-type animals, in both the prenatal and adolescent exposure groups. There was no significant difference for any water types, indicating metal enhancement may affect novel object recognition. However, unexpectedly, the results showed that animals raised on iron-enhanced water performed better than lab tap water animals. In group 1, Tg lab animals failed to show object recognition while Tg iron animals maintained object recognition. In group 2, iron animals sniffed more than lab animals on either the standard or the novel object, regardless of genotype. Also unexpectedly, there was no change in object recognition due to either initial prenatal or adolescent exposure to iron. Although increased exposure to iron did not impair novel object recognition, it did diminish the level of sniffing. This suggests that regardless of the exposure time to iron-enhanced water, there is evidence to suggest a remediation effect of recognition memory for these mice due to iron enhancement. For both groups, plaque load was dependent on region of interest with higher plaque burden in the hippocampus. This was expected as the hippocampus is one of the first areas of the brain to develop plaques in AD. Overall, these results indicate that NOR can be used to assess transgenic mice and that iron may exhibit a rescuing effect for recognition memory deficits caused by the Alzheimer's disease.

CHAPTER 1: INTRODUCTION

Alzheimer's disease (AD) is the seventh leading cause of death in the United States (Center for Disease Control and Prevention [CDC], 2008) with the personal cost of care for an AD individual reaching an average of $174,000 for the progression of the disease (National Institutes of Health [NIH], 2008). There are 360,000 people diagnosed with AD each year (The Brain Matters.org, 2009) with a current estimate of 4.5 million individuals in the American population diagnosed with Alzheimer's disease overall (Herbert, Scherr, Bienias, Bennett, Evans, 2003; NIH, 2008). 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 et al., 2003). The Alzheimer's Association and the National Institute on Aging have both reported that the national, direct and indirect, annual costs of caring for individuals with AD are at least $100 billion (NIH, 2008). 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, 2007).

There is currently the largest growing senior citizen population in America with the longest life expectancy ever experienced. The population of Americans aged 65 or older, the age at which one is considered a senior citizen, will double over the next 25

years. Thus, roughly 20 percent of the U.S. population, or 71 million Americans, will be considered senior citizens by 2030. The baby boomer population of Americans born between 1946-1964 will start to reach age 65 by 2011. Age-related diseases and disorders are becoming more prevalent than ever before simply because people are living longer. This makes fields of research pertaining to diseases that afflict the elderly increasingly more important. In addition, with the intention of achieving a health benefit, there are more 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, enhancement of all types of food and drinks (e.g., bread, orange juice, milk), and incidental exposure (e.g., environmental exposure, tap water) are all potential sources of overloading our systems in an attempt to be healthier.

Deficits of Alzheimer's disease

To study possible prevention methods for Alzheimer's disease, there must be an understanding as to the brain pathology and behavioral symptoms that accompany the disease. The major known protein components of plaques and tangles are amyloid beta protein precursor and the microtubule-associated protein tau, respectively. In AD, plaque and tangle formation can be found distributed throughout the brain alone or together in a variety of brain regions; however, the relationship between plaques and tangles remains a controversial issue in the AD field (Goedert and Crowther, 1999). After amyloid-β (Aβ) is created (by cleavage of APP, discussed later in the "*Trace Metals and their Physiological Function in the Brain*" section) it is released as a soluble peptide that may deposit and begin aggregation in the neuropil. Once aggregation of Aβ has begun there

appears to be induction of free radical damage to neurons (Kosik, 1994). The mechanism of Aβ formation has not been completely identified; though, it does appear there is neuronal damage prior to Aβ deposition (Goedert and Crowther, 1999). Amyloid-β is a metalloprotein which is also comprised of zinc, copper, and possibly iron (Opazo, Huang, Cherny, Moir, Roher, White, et al., 2002). Neurofibrillary tangles (NFTs) consist of highly ordered intraneuronal filaments called PHF assembled from tau. Within neuronal cell bodies and neurites lie NFTs, where processes are induced to become swollen and dystrophic. The tau protein of these NFTs play a role in neuronal degeneration through the disassembly of cytoskeletal systems, which includes microtubules and neurofilaments, along with the loss of other structures (i.e., synapses) (Kosik, 1994). Essentially, the proteins that make-up plaques and NFTs become twisted and tangled together causing damage to the surround brain tissue and cells.

It is also important to understand the relationship between the type of memory and brain regions involved for both tasks of recognition memory (which will be discussed and tested in this paper) and the progression of Alzheimer's disease pathology. Declarative memory, what is commonly thought of when "memory" and "remembering" are referred to in ordinary language, is the basis for conscious recollection of facts and events. Specific brain systems, and some surrounding cortex, are required for declarative memory; in particular the hippocampal formation is required. The use of declarative memory supports the rapid learning of novel associations (Squire, 1992). Declarative memory is comprised of fact (semantic) and event (episodic) memory. Studies of amnesiac patients have shown that the formation of context-free semantic memory is

supported by the peripheral and entorhinal cortices, but cannot support context-rich episodic memories alone. Episodic memories must require the additional processing power provided by the hippocampal circuit (Vargha-Khadem, Gadian, Watkins, Connelly, Van Paesschen, Mishkin, 1997). Non-declarative memory reflects several non-conscious forms of learning including information acquired during skill learning, non-associative learning (i.e., habit formation), classical conditioning, priming. These forms of memory are expressed through performance (overt responding) rather than conscious recollection. The rapid learning of novel associations is not typically supported for skill learning, but this type of memory may be appropriate for gradual and cumulative acquisition of new associations. Research indicates that multiple brain systems are involved for different forms of non-declarative memory; and while the hippocampal formation is not required for these forms of memory, the basal ganglia is required for skill learning(Squire, 1992).

Alzheimer's disease follows a classic pattern of pathological progression through the brain characterized by amyloid plaques (plaques) and neurofibrillary tangles (tangles). Development of plaques and tangles can first be found in the entorhinal cortex and then spread to the hippocampal area. The damage further progresses to the amygdala, over-lying cortices of the brain, pre-frontal lobe, and eventually to the sensory and motor areas. This course of development of these pathologies is consistent with the cognitive declines seen through the advancing stages of Alzheimer's disease. The entorhinal cortex is linked to semantic knowledge which is impaired in the early stages of the disease (i.e., patients may not be able to name or ask for objects they are directly

looking at with the correct object name). The hippocampal region is essential for episodic, or event, memories which are often seen as a deficit that starts with an inability to recall recent events. The hippocampus also plays an important role in spatial memory, or the ability to record and recall information about the environment and determine spatial orientation (i.e., patients may easily lose their way going to familiar places). Emotions are affected and become less controllable as the amygdala, responsible for aspects of emotional memory of the brain, develops hallmark lesions. Lack of regulation from the pre-frontal cortex will also affect the ability to control and regulate emotions. The ability to store new memories (working memory) as well as to retrieve older memories is further compromised when the over-lying cortices and the pre-frontal lobe become affected. As plaques and tangles develop in the sensory and motor areas, motor functions and sensory discrimination will rapidly decline in the final stages of the pathological progression of the disease.

Decline in recall and recognition, thought processing, use of words, and behavior patterns are all common, visible signs of Alzheimer's disease (AD). Cognitive changes resulting from AD are seen to develop in three stages: mild, moderate, and severe AD. Mild AD starts with an impaired ability for consolidation and decreased capacity for short term memory, trouble with thought organization and logical thinking, increased likelihood of getting lost or forgetting how to get to a familiar place, and increasingly resisting change to new things. Moderate AD is characterized by increasing changes in behavior and frequency of forgetful episodes, trouble recognizing familiar people and objects (personal belongings), decreased ability for activities of daily living (ADLs),

decreased ability to differentiate sensory input, and an increase in repetition of thoughts, stories, words or motions. Lastly, symptoms of severe AD are classified by the inability to recognize close family and friends and/or self, speaking in gibberish, becoming difficult to understand, or becoming mute, a total loss of ADLs (e.g., forgetting how to make toast or how to put on clothes in the proper order), or appearing to be stuck in the past due to severely diminished memory recall (AD Education & Referral Center [ADEAR], 2009).

Trace Metals and their Physiological Function in the Brain

Alzheimer's disease (AD) is a polygenic neurodegenerative disorder involving the copper-zinc metalloprotein amyloid-β and its abnormal accumulation and deposition into plaque aggregates (Maynard, Bush, Masters, Cappai, Li, 2005) (Fig. 1). The zinc model finds that plaques exist within a mix of free Zn^{2+} and soluble Aβ (bound to Cu^{2+} or Fe^{2+}). This model for Alzheimer's disease suggests that "amyloid neuropathy of AD is principally caused by zinc released during neurotransmission (Bush and Tanzi, 2005, p. 7317)." Factors that affect β-amyloid plaque formation are seen in Fig. 1. Harmful hydrogen peroxide (H_2O_2) is produced by both Aβ and plaques. This excess H_2O_2 can inhibit the ability of biochemical factors and chelating drugs to lower the concentration of free zinc. (These biochemical factors include apolipoprotein E (ApoE), α-2-macroglobulin (α2M), metallothioneins (MT) and chelating drugs include clioquinol (CQ) Bush and Tanzi, 2005).)

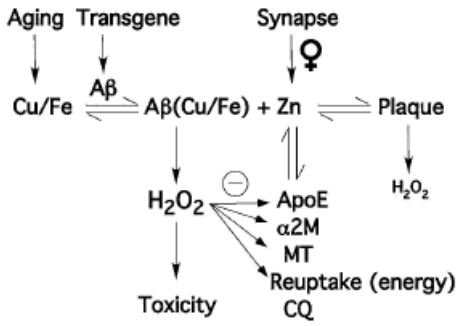


Figure 1: Zinc hypothesis of Alzheimer's disease. (Bush and Tanzi, 2005)

Through the process of proteolysis, amyloid precursor protein (APP) gives rise to 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, Moir, Huang, Scarpa, Bacarra, Romano, 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 nonamyloidogenic pathway which prevents Aβ production after cleavage by α-secretase within the APP sequence (Selkoe, 1998) (Fig. 2).

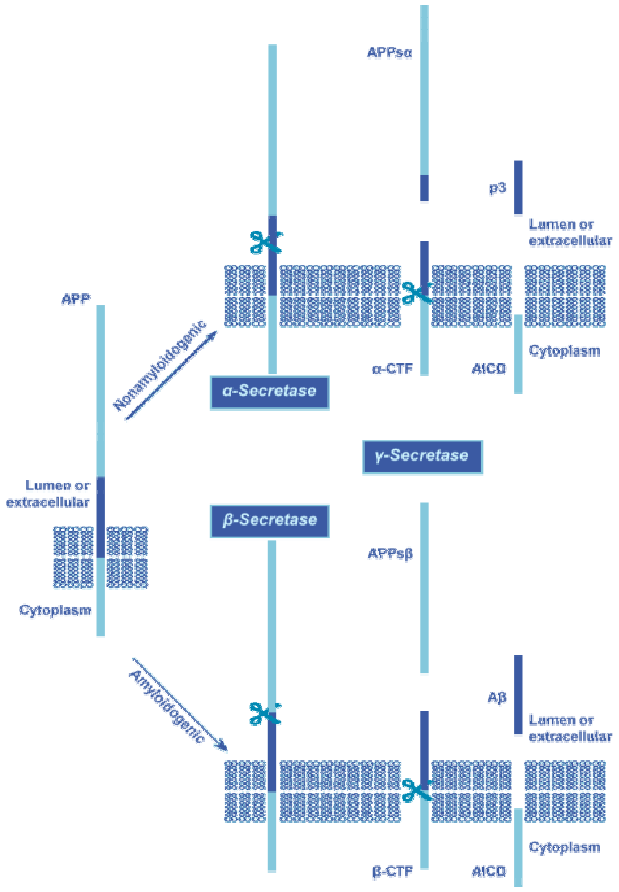


Figure 2: Proteolytic processing of the amyloid precursor protein (APP) by secretases. (European Iron Club, 2007)

Certain risk factors, specifically exposure to metals such as aluminum, iron, and zinc, may contribute to AD by accelerating the aggregation of Aβ (Esler, Stimson, Jennings, Ghilardi, Mantyh, Maggio, 1996), which has been proposed as a critical event in the etiology of the disease (Hardy and Allsop, 1991; Joachim and Selkoe, 1992). Aluminum, iron, and zinc cations are found to significantly enhance the rate of aggregation of physiological concentrations of Aβ (Mantyh, Ghilardi, Rogers, DeMasters, Allen,

Stimson, et al., 1993). In the presence of copper an increase of α-secretase cleavage is seen leading to an increase of the nonamyloidogenic APP fragment. Hence, copper appears to promote the nonamyloidogenic pathway of APP and may be a useful tool in the search for anti-Alzheimer drugs (Borchardt, Camakaris, Cappai, Masters, Beyreuther, Multhaup, 1999). Lovell, Robertson, Teesdale, Campbell, and Markesbery (1998) have since 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.

Zinc, copper, and iron are considered biochemically functional metals, or biometals, which are known as trace metals in the neuroscience literature. However, these metals cannot be considered either trace in concentration or irrelevant and in fact partake in many essential activities of the brain and body. 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). The highest level of vesicular zinc can be found in the ultrastructure of the mossy fiber boutons, essential to memory functions. This zinc (Zn^{2+}) is released during glutamatergic neurotransmission (Bush, 2000). There is re-uptake of the zinc into the post synaptic CA3 neurons and mossy fiber terminals for the initiation of mossy fiber long term potentiation (LTP) (Takeda, Kanno, Sakurada, Ando, and Oku, 2008).

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). 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 a necessary constituent for cytochrome oxidases, numerous enzymes in the citric acid cycle, ribonucleotide reductase, and NADPH reductase (Wigglesworth and Baum, 1988). Iron is fundamental to oxygen transport as it is the central molecule in the oxygen transport structure and system. The hemoglobin molecule is comprised of four globular protein subunits, each of which contains a non-protein heme unit (Steinberg, Forget, Higgs, Nagel, 2001). At the center of the heme group lies an iron ion held in a heterocyclic ring, in part, consisting of four nitrogen ions. Oxygen binds to the iron ion, which is present and can bind in either the Fe^{2+} or Fe^{3+} state; However, oxygen will not bind with methemoglobin (Fe^{3+}) (Linberg, Conover, Shum, Shorr, 1998). 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 fundamental 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). Thus iron and copper form sections of enzymes required for indispensable brain function including energy production, neurotransmitter synthesis, and antioxidant function.

Trace Metals in Alzheimer's disease

In Alzheimer's disease the balance of brain metals shifts. Certain metal ions (copper, iron), through electron donation/acceptance, have the ability to lead to radical

formation, ROS (reactive oxidative species), and oxidative attack on tissue components, which may contribute to disease. In contrast, zinc maintains valence and acts as an antioxidant, but is still linked to increased Aβ precipitation through plaque aggregation. In both humans and transgenic mice, iron can be found to be concentrated in the area surrounding amyloid plaques. Copper and zinc directly bind to the Aβ that compromises amlyoid plaques. 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). Barnham and Bush (2008) assert that "the consensus that has emerged is that zinc and copper are enriched in amyloid where they coordinate Aβ, iron is enriched in the tissue and neuritic pathology, and there is evidence of functional copper deficiency" (p. 225).

APP (Bush, Martins, Rumble, Moir, Fuller, Milward, et al., 1990) and zinc (Baker, McNeil, Lander, 1978; Frederickson, 1989) can be found in high concentrations in platelets and in the brain in the normal person, with the highest concentrations of zinc found in the cerebral cortex, particularly the hippocampus (Frederickson, Klitenick, Manton, Kirkpatrick, 1983). Zinc homeostasis in the brain becomes abnormal (Bush, Pettingell, d. Paradis, Tanzi, 1994a) when zinc concentration gradients are negatively affected by the pathological lesions of AD (Hyman, Van Hoesen, Kromer, Damasio, 1986; Glenner and Wong, 1984). These lesions most severely and consistently affect the hippocampus (Bush et al., 1994a). Elevated (80%) cerebral spinal fluid zinc levels (Hershey, Hershey, Varnes, Vibhakar, Lavin, Strain, 1983), increased extracellular Zn^{2+}-metalloproteinase activities in the AD hippocampus (Backstrom, Miller, Tökés, 1992), and decreased concentrations of astrocytic growth inhibitory factor, a protein which

naturally chelates zinc (Uchida, Takio, Titani, Ihara, Tomonaga, 1991), can be incorporated as evidence for altered zinc metabolism in AD. Bush et al. (1994a,b) have proposed that together this evidence could be indicative of an abnormality in the uptake or distribution of zinc in the AD brain.

By modifying its adhesiveness to extracellular matrix elements, extracellular zinc may play a role in the physiology of APP function (Bush, Multhaup, Moir, Williamson, Small, Rumble, 1993). Abnormal zinc homeostasis, due to hippocampal lesions, might explain the high affinity of zinc chelation due to Aβ. The chelation of zinc is so high that it has been shown to produce neurotoxic effects in neuronal cultures (Yankner, Duffy, Kirschner, 1990; Koh, Yang, Cotman, 1990). These findings are supported by Bush et al. (1994a) which demonstrated that the physiological concentrations of zinc increase the resistance of the APP peptide to proteolytic catabolism and promote Aβ precipitation. It is hypothesized that "zinc-induced accumulation of Aβ in the neuropil may, in turn, invoke a glial inflammatory response, free radical attack, and oxidative cross-linking to form an, ultimately, 'mature' amyloid (Bush et al., 1994a, p. 12157)." Therefore, in cases of Alzheimer's disease, excessive zinc concentrations may serve to accelerate Aβ deposition (Bush et al., 1994a).

Bush et al. (1994a) have shown that over-production of soluble Aβ (soluble and insoluble forms of Aβ will be discussed later) cannot sufficiently explain Aβ precipitation, leading to the hypothesis that biochemical mechanisms which promote Aβ formation may be relevant to the pathogenesis of Alzheimer's disease. Increased concentrations of zinc promote the Aβ peptide's adhesiveness and resistance to

proteolytic digestion, which may be important for the metabolic fate of Aβ and cerebral zinc homeostasis. High extracellular and low intracellular concentrations of cerebral zinc are caused by an abnormality in the uptake and distribution of zinc in the brain of AD patients (Bush et al., 1994a). The effects of physiological concentrations of zinc on the stability of synthetic human $A\beta_{1-40}$ in solution and its effects to those of the rat-mouse Aβ species were examined (Bush, Pettingell, Multhaup, d. Paradis, Vonsattel, Gusella, et al., 1994b). Rat neuronal tissue produces soluble $A\beta_{1-40}$ (Busciglio, Gabuzda, Matsudalra, Yankner, 1993); however, $A\beta_{1-40}$ amyloid deposition is not a feature of normal aged rat brains. This research (combined with Bush et al., 1994a) shows that soluble human $A\beta_{1-40}$ has a greater tendency than rat $A\beta_{1-40}$ to form amyloid in the presence of physiological zinc concentrations. This vulnerability of human Aβ to zinc-induced amyloid plaque formation is a convincing explanation for human susceptibility to (pathology of) AD.

Amyloid-β species of varying conformations may play differing roles in AD, causing neurotoxicity in the brain by distinct mechanisms that affect neuronal functions. Species include amyloid β oligomers (AβO), Aβ-derived diffusible ligands (ADDLs), and fibrillar Aβ (Aβf). Soluble Aβ is comprised of intermediate Aβ species including ADDLs, AβOs, protofibrils (or string of oligomers) and dodecomeric oligomers Aβ*56. Through conformational changes (i.e., trimer, pentamer, or higher molecular weight complex formation) fibrilization occurs and the insoluble form of Aβ is formed (Aβf). The fibrillar, or insoluble, form of the Aβ peptide is the primary conformation of senile plaques. Exposure of neurons to Aβf induces neuritic dystrophy and synaptic loss

through the synaptic alterations; however, high concentrations are required to produce wide-spread neuritic dystrophy (Deshpande, Mina, Glabe, Busciglio, 2006).

AβOs (Aβ oligomers or soluble Aβ) bind rapidly to synaptic contacts and cellular membranes, with high affinity, and induces rapid and massive neuronal death through apoptosis and loss of cell viability. Parameters used to measure neuronal death include decreased mitochondrial metabolic activity, increased capsase activity, and increased levels of lactate dehydrogenase (LDH). AβO can be neurotoxic in low concentrations, can induce inhibition of long-term potentiation, and cognitive dysfunction (measured in part through neuronal death). Importantly, the severity of cognitive impairment correlates better with brain levels of soluble Aβ species than the density of plaque deposition (Deshpande, Mina, Glabe, Busciglio, 2006).

Zinc enhances the tendency for aggregation (adhesiveness) of the $A\beta_{1-40}$ peptide to produce aggregates with the staining/coloring properties of amyloid plaques with the ability to create zinc-induced tinctoral amyloid formations (Bush et al., 1994a,b). Tinctorial amyloid formation criteria, or conformation properties which allow light reflection and staining, can be checked when the zinc-induced Aβ aggregates are stained with Congo red and put under polarized light to ensure they manifest a green birefringence (Glenner, 1980). Clements et al. (1996) looked at the differences in aggregation of three different formations/mutations of Aβ peptide in an attempt to find a relationship between the aggregation differences due to metal binding. These mutations do not materially alter the binding of metal, leading to the idea that the binding region is unlikely to be in the immediate vicinity of the mutation sites. The resulting data thus

suggests that while amyloid plaques bind to zinc, binding is not confined to just zinc; Cu^{2+} may have a significant affinity for these peptides as well, and given the ability of Cu^{2+} to displace Zn^{2+}, the Zn^{2+} binding site appears to be highly attractive to the copper ions (Clements et al., 1996). These findings suggest that between zinc and copper there may be competitive binding in vivo for the binding sites on amyloid peptides, leaving open the possibility for future research of zinc binding to amyloid peptides through the investigation of the structural make-up of the metal-peptide complex.

Aβ is markedly precipitated by certain metals, particularly Zn^{2+}, in vivo (Bush et al., 1994 a, b; Bush, Moir, Rosenkranz, Tanzi, 1995; Huang, Atwood, Moir, Hartshorn, Vnsattel, Tanzi, Bush, 1997). Early work showed that salts of aluminum (Al), iron (Fe), and zinc (Zn) significantly enhance the rate of Aβ aggregation in vitro, at concentrations of $\geq 10^{-6}$M. Zinc appears to be the most effective metal, clearing a large majority of Aβ, at physiological concentrations, from solution, in a control Tris-HCL buffer or human CSF (Mantyh et al., 1993). However, Bush et al. (1994b) reported comparable levels of Aβ aggregation in response to lower zinc concentrations than those previously reported. Since zinc and other biometals are concentrated in the brain neocortex this is particularly important when there is Aβ formation in the same region. Further evidence has been found that zinc and copper contribute to the deposition of Aβ within amyloid plaques, through the examination of direct application of zinc and copper chelators to brain tissue (discussed in "Chelation of Brain Metals" section) (Cherny, Masters, Beyreuther, Tanzi, Fairlie, Bush, 1997; Cherny, Legg, McLean, Fairlie, Huang, Atwood, et al., 1999).

With Alzheimer's disease, altered neuronal H^+ homeostasis may be seen along with the appearance of amyloid deposits. This leads to an increased acidodic state of the AD brain. Cerebral acidosis may complicate AD (Yates, Butterworth, Tennant, Gordon, 1990) which may be related to the inflammatory response seen in AD brain tissue (Griffen, Sheng, Mrak, 1997; Rogers and O'Barr, 1997). The amyloid-β peptide catalyses the reduction of Cu^{2+} to Cu^+ and Fe^{3+} to Fe^{2+}, generating hydrogen peroxide (H_2O_2) from molecular oxygen (O_2) (Opazo, et al., 2002). The interaction of the peptide with copper and iron mediates the toxicity of the amyloid-β peptide by producing toxic hydrogen peroxide, which will further react with the reduced metal ions to generate toxic hydroxyl radical [Fenton reaction (Maynard et al., 2005)]. Atwood et al. (1998) looked for any interactions that might help explain the tendency for Aβ to precipitate under conditions expected, mildly acidotic (pH = 6.6, 6.8 and 7.4), within the metabolically diseased brain parenchyma. The effects of several bioessential metal ions were investigated, including Zn^{2+}, Fe^{3+}, and Cu^{2+}, along with their ability to bind to and alter the solubility of various forms of Aβ. Zn^{2+} and Cu^{2+} induce 30% more aggregation, with copper causing the most striking increase in Aβ aggregation (Atwood et al., 1998). These results reaffirmed the importance of the Aβ:Cu^{2+} interactions to the pathophysiology of amyloid deposition (Cherney et al., 1999).

Beard, Connor, and Jones (1993) believe that dysfunction regulation of the proteins responsible for iron storage and transport results from iron imbalance. Comparing normally aged brain tissue and comparable locations of AD brain tissue, transferrin concentration is found to be lower, ferritin levels are either unchanged or

slightly unchanged, and iron levels are higher (Connor, Boeshore, Benkovik, 1992a). This suggests increased storage of iron per mol of ferritin and decreased mobility of iron. The chances of iron-induced peroxidative damage due to the boost of iron are increased because iron can readily be removed from ferritin by a reducing agent (Connor and Fine, 1986). 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, but is the least studied regulatory protein (Hallgren and Sourander, 1958; Octave, Schneider, Touet, Crichton, 1983). 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 and Benkovic, 1992). This has important implications for the effect of iron overload on neuronal development and communication.

An example of dangerous iron overload pathology is Hallervorden-Spatz syndrome, an iron deposition and overload disorder characterized brain degeneration due to the overload. This disease has also been termed “neurodegeneration with brain iron accumulation type1" (NBIA-1) (Neumann, Adler, Schluter, Kremmer, Benecke, Kretzschmar, 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).

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, Scarpa, Huang, Moir, Jones, Fairlie, 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). In both humans and mice, a significant increase of iron levels in the brain with age has been repeatedly shown (Drayer, Burger, Darwin, Riederer, Herfkens, Johnson, 1986; Thomas, Boyko, Anthony, Burger, 1993; Bartzokis, Beckson, Hance, Marx, Foster, Marder, 1997; Martin, Ye, Allen, 1998; Zecca, Gallorini, Schunemann, Trautwein, Gerlach, Riederer, et al., 2001; Maynard, Cappai, Volitakis, Cherny, White, Beyreuther, et al., 2002). Since levels have been shown to rise in the aging mouse brain, it can be suggested that levels of copper in the human brain probably also increase with age (Massie, Aiello, Iodice, 1979; Morita, Kimura, Itokawa, 1994; Maynard et al., 2002).

In AD-diseased brains, the levels of both iron and copper ions that increase with age in the non-diseased brain are even further elevated (Wender, Szczech, Hoffmann, Hilczer, 1992). Reports of changes in brain zinc levels during the aging process have been limited and inconsistent (Maynard et al., 2005), though it appears that zinc levels in the brain remain either unchanged or show a slight decrease with age, as seen in results from experiments with mouse brain tissue (Woodward, Filteau, Allen, 1994; Morita et al., 1994) and human serum (Bohnen, Jolles, Degenaar, 1994; Del Corso, Pastine, Protti,

Romanelli, Moruzzo, Ruocco, et al., 2000). 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, LeWitt, Juneau, Sima, Nguyen, DeMaggio, et al., 1996; Castellani, Smith, Nunmura, Harris, Perry, 1999; Maynard et al., 2005).

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. 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. Could these other mechanisms be linked to the increases in all of 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, DeMaggio, Juneau, Brickman, Mashour, Fickelman, 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). Through specific receptors located on the brain microvasculature (Jefferies, Brandon, Hunt, Williams, Gatter, Mason, 1984), transferrin is responsible for delivering iron across the blood-brain barrier (Fisherman, Rubin, Handrahan, Connor, Fine, 1987; Partridge, Eisenberg, Yang, 1987). Neurons reportedly receive iron by means of a transferrin-mediated process as it crosses the blood-brain barrier (Swaiman and Maxhen, 1985). Zinc CSF levels are also elevated by eighty percent when zinc concentration gradients are affected by AD (Hershey et al., 1983).

With Alzheimer's disease there is a change in not only the structural pathology (plaques and tangles) and behavioral symptoms, but in the overall brain chemistry. Levels of APP and Aβ are changed as well as metal concentrations (zinc, copper, iron) n both the brain and CSF. These changes in metal chemistry could be key to novel therapies (e.g., chelation) and treatments for Alzheimer's disease.

Chelation of Brain Metals

Metal chelation has been proposed as a therapy for AD. This chelation could have the potential to dissolve precipitated Aβ peptides, which comprise one of the pathological hallmarks of the disease and are comprised of multiple metals, and prepares them for possible removal from the brain. Chelation of the metals bound to and associated with the Aβ peptides could provide a reversal of the behavioral effects of AD through the dissolution of the pathogenic changes in the brain. 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, Collingwood, Khan, Korchazkina, Berthon, Exley, 2004). There are several chelators which are currently being tested as potential therapies, which include desferioxamine mesylate (DFO), ethylenediamineletraacetic acid (EDTA), Feralex-G (Feralex), and Clioquinol (CQ).

DFO has been shown to be an effective chelator of amyloid both pre- and post-formation, effectively preventing and reversing the formation process. This compound chelates both aluminum and iron. DFO forms complexes with trivalent metal ions (Al (III), Fe (III)) producing a loosening of plaque-like structures and preventing the formation of amyloid fibrils in β-pleated sheet conformation (House et al., 2004). In a

human study of patients with AD, DFO produced a reduction of pathological brain aluminum, back to normal human adult cortical levels (Kruck, Krishnan, McLachlan, Percy, 2002). Additionally DFO has been moderately effective in the reduction of mental deterioration of patients with AD (Crapper-McLachlan, Dalton, Kruck, Bell, Smith, Kalow, & Andrews, 1991).

EDTA can chelate copper alone, which is not beneficial as copper is considered as protective and must only be removed at the same time as zinc, iron, and/or aluminum; thus preventing formation of Aβ or initiating harmful dissolution of fibrillar Aβ (House et al., 2004). Feralex has exhibited the ability to chelate both iron and aluminum. Feralex removes hyperphosphorylated ι (PHF-ι) bound to Al (III) and Fe (III). In a comparison of Feralex to DFO, both compounds equally chelated iron, but Feralex showed increased efficiency in aluminum chelation (Shin, Kruck, Murayama, Kitamoto, 2003). This suggests that Feralex could prove to be a stronger, more efficient chelator than DFO which may be more beneficial in clinical applications.

Clioquinol (or iodochlorohydroxyquinolin) is a quinoline chelator which inhibits the precipitation of Aβ by Cu^{2+} and Zn^{2+} and rescuing neuronal cell cultures from Aβ toxicity. CQ uses a more novel mechanism that does not act through a non-specific brain metal depletion mechanism as in traditional chelation therapy and may even included reestablishment of normal metal homeostasis (Opazo, Luza, Villemagne, Volitakis, Rowe, Barnham, et al., 2006). The mechanism of CQ has been discerned into 3 steps: (1) Targeting, in which CQ enters the brain to form a ternary complex with metallated Aβ, (2) Transfer, in which CQ abstracts the Cu^{2+}/ Zn^{2+} from Aβ, and (3) Redistribution, in

which CQ delivers Cu^{2+}/Zn^{2+} to other metal binding ligands or proteins. After these steps have occurred, CQ is cleared into the plasma without the necessity of promoting the excretion of metal (Opazo, et al., 2006). It has been shown to improve neurological scores of treated animals (Tg2576 mice) while clearing amyloid deposition (Cherny, Atwood, Xilinas, Gray, Jones, McLean, et al., 2001). Additionally an oral administration pilot study of AD patients showed significantly slowed cognitive decline compared to placebo controls. Using the baseline cognitive subscale scores on the Alzheimer's Disease Assessment Scale, researchers observed a worsening of scores from patients in the placebo group while minimal deterioration was exhibited in the CQ group (Opazo, et al., 2006).

While chelation has shown promise as a potential therapy for Alzheimer's disease, the technique has not yet shown the capability for wide-spread use. One issue of concern in translation from chelation research to Alzheimer disease prevention is which metal is the most beneficial to have removed from the diseased brain. DFO and Feralex remove both aluminum and iron, which show several similar physiochemical features (Shin et al., 2003) while EDTA and CQ can remove copper and zinc. Current chelators appear to lack the ability to remove only a single metal from the brain. This provides a need for comprehensive, individual metals research to determine how each metal plays a role in influencing AD.

Transgenic Models of Alzheimer's disease

Definitive diagnosis of human AD is made postmortem using amyloid plaques for a pathological diagnosis. In patients with AD, large numbers of senile plaques are found

in the brain. These senile plaques are composed of amyloid-beta (Aβ) protein that is derived from amyloid precursor protein (APP), the larger original protein (Hsiao, Chapman, Nilsen, Eckman, Harigaya, Younkin, et al., 1996). Prior to postmortem diagnosis, a general diagnosis of dementia of the Alzheimer's type can be made based on cognitive deficits and memory loss. This cognitive deterioration has been linked to the increase of amyloid in the brain, leading to an increase in senile plaques accumulating throughout the brain (Hsiao Ashe, 2001). There is some research that shows that individuals may have a high plaque load, yet remain cognitively normal (Katzman, Terry, DeTeresa, Brown, Davies, Fuld, et al., 1988; Dickson, Crystal, Mattiace, Masur, Blau, Davies, et al., 1992); and although these cases should be examined, they are the exception to the general correlation between increased amyloid production/accumulation and cognitive deficit decline.

To study the effects of amyloid on AD as well as other factors that contribute to the aggregation of amyloid plaques, animal models must be used. The strongest of the current transgenic mouse models are APP transgenic or APP/PS1 (presenilin 1) bigenic mice. The APP gene naturally carried by mice does not produce Alzheimer's disease pathology, so knock-in genes are used to create hAPP (human amyloid precursor protein) mouse lines that can be crossed with other transgenes. These models result in both amyloid deposition and progressive memory loss which are pathological and behavioral hallmarks of Alzheimer's disease. These mouse models are missing neurofibrillary tangles, which present a problem in their absence for allowing the full study of the disease but allows for the individual study of amyloid plaques. 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, Lemere, Capell, Citron, Seubert, Schenk, 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 (Hisao et al., 1996) and T-maze (Chapman, White, Jones, Cooper-Blacketer, Marshall, Irizarry, et al., 1999).

Dietary manipulations which may cause cognitive deficits and negative changes in brain pathology cannot be tested on diseased (i.e., Alzheimer's afflicted patients) and non-diseased humans. To test the effect of an overload of metals in the diet, transgenic animals must be used and the findings translated to fit a human diagnosis. This translation from animal modeling to humans is made through behavioral testing which models human behaviors as closely as possible.

Behavioral Testing of Alzheimer's disease Mice

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 visual paired-comparison (VPC) task is a test of recognition memory that is similar to the novel object recognition (NOR) task which is also a test of recognition memory. The VPC is sometimes used to assess visual-recognition in monkeys and infant humans. An example VPC task paradigm would consist of a presentation phase, a priming test five

minutes later, and a yes/no recognition memory test 24 hours later. During the presentation phase participants see pairs of identical pictures (A-A) presented simultaneously, then during the priming test, participants see 24 old-new pairs of pictures or words (A-B). The yes/no recognition memory test consists of 48 items with old pictures (A) and new pictures (C) presented individually and participants are asked to show whether they have seen the picture previously or not (Manns, Stark, Squire, 2000). Although similar to the rodent NOR task, the VPC is reflective of a type of visual memory, while it is less clear if NOR can be definitively described as a visual object recognition task. However, NOR can certainly be used in the study of object-recognition memory. Due to this difference, direct comparisons of findings with rodents in the NOR to findings in the VPC with infants and monkeys should be made cautiously (Mumby, 2001).

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. Little research has been conducted to link brain pathology (amyloid plaques) to behavioral results of AD related cognitive deficits (e.g., results of the NOR task).

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.

Hypothesis/Specific Aims

We know that after an Aβ aggregate is formed, growth of the aggregate can and will continue with even a low concentration of the Aβ peptide (Maggio, Esler, Stimson, Ghilardi, Allen, Dahl, et al., 1992). Since research has shown that an aggregate can be easily sustained once formed, the next question becomes what (risk) factors promote

initial Aβ aggregation? Esler et al. (1996) provided evidence that zinc, in relatively high concentrations, is able to induce the initial aggregation of Aβ. A paradoxical effect appears due to dietary enhancement of copper: while replenishment of deficient copper may "decrease the proportion of APP that undergoes amyloidogenic processing, concurrent upregulation of APP expression may add to net Aβ burden (Maynard et al., 2005)." A balance must be attained in the attempt to restore copper deficiencies and normal enzyme activity and the possibility of increasing APP expressing with toxic Aβ oligomer formation. If high concentrations of zinc and copper in the brain can induce Aβ aggregation, then what is the possible effect of an overload of dietary enhancement of these metals on the brain and brain function?

There is a large body of research that investigates the biochemical aspects of the amyloid precursor protein, Aβ aggregation, and the effects of trace metals (copper, iron, zinc) at the cellular level. However, this field is lacking in a way to link the physiological mechanisms to the physical and personal expression of the disease in a behavioral manner. To investigate the direct effects of metals on Alzheimer's disease (using an animal model), a task which measures aspects of learning and memory should be used to help link these two aspects together. We examined the individual effects of specific metals (zinc, copper, and iron) to see their effect on learning and memory in mice, using the NOR test.

Aim One

The first aim of this study was to demonstrate that novel object recognition can be used as a standard task for measuring (impairment of) recognition memory in transgenic

mice. It has been shown that the NOR task is a reliable measure for the assessment of object recognition in non-transgenic mice, rats, and other mammals (Mumby, 2001). Tg2576 mice show cognitive deficits between 3 to 15 month (Hisao Ashe, 2001) and have shown deficits in spatial tasks such as the Morris water maze (Hisao et al., 1996). We hypothesized impairment of recognition memory, as assessed through the novel object recognition task, in transgenic (Tg) type animals over wild-type (Wt) animals (hypothesis 1).

Aim Two

The second aim of this study was to examine the effects of prenatal versus adolescent exposure to iron enhanced water along with the effect of the AD transgene on learning and memory. 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). We hypothesized that animals raised on iron enhanced water from birth (prenatal) would show increased deficits in recognition memory relative to animals raised on iron enhanced water beginning at 3 months of age (adolescence), with animals raised on lab tap water performing the best on these tasks (hypothesis 2a). We further hypothesized that memory deficits would be more severe in iron enhanced water animals of the Tg and Wt types than in the lab water animals of the Tg and Wt types (hypothesis 2b).

Aim Three

The third aim of this study was to examine the effects of zinc and zinc plus copper enhanced water along with the effect of the AD transgene on learning and memory. We

hypothesized that zinc would affect learning and memory in animals and therefore, animals raised on zinc enhanced water compared to lab tap water would show decreased performance on behavioral measures (hypothesis 3a). We further hypothesized that recognition memory deficits would be more severe in zinc animals of the Tg and Wt types than in the lab water animals of the Tg and Wt types (hypothesis 3b). Clements et al. (1996) showed that copper is the most effective metal (>95%) in its ability to displace zinc, compared to other metal ions examined. It has also 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). This statement is supported by the fact that 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, Schäfer, Simons, Kemmling, Kamer, Tepest, et al., 2003) and mutant alleles (Phinney, Drisaldi, Schmidt, Lugowski, Coronado, Liang, et al., 2003). We hypothesized that animals raised on zinc plus copper enhanced water would show increased performance on learning and memory behavioral tasks than animals on zinc enhanced water, but not as well as animals on lab tap water (hypothesis 4a). We further hypothesized that memory deficits would be less severe in zinc plus copper animals of the Tg and Wt types than in zinc animals of the Tg and Wt types, but the least severs in the lab water animals of the Tg and Wt types (hypothesis 4b).

CHAPTER 2: METHODS AND DATA ANALYSIS

This study involved comparisons of different metal enhanced water groups. The first comparison examined the effects of iron enhanced water consumption beginning in pre-adolescence versus consumption beginning during adolescence. Learning and memory was assessed through the behavioral task of novel object recognition (NOR). The second comparison examined the effects of zinc enhanced, zinc plus copper enhanced, and lab tap water on memory and learning through NOR. Animals with initial iron enhanced water exposure beginning prenatally came from group one and animals with initial iron enhanced water exposure beginning during adolescence came from group two. All animals from the second comparison of zinc, zinc plus copper, and lab tap water came from group two. Groups one and two will be discussed in *Experimental Animals* (Fig. 3).

Experimental Animals

Animal subjects used in this experiment were transgenic (Tg) and wild-type (Wt) APP2576 mice. These are transgenic mice that carry the human amyloid precursor protein (APP) mutation, a model developed by Karen Hsiao (Hsiao et al., 1996). Group one consisted of sixteen Tg2576 and eighteen Wt2576 mice raised on either lab tap water (n = 18) or iron enhanced water (n = 16) from beginning prenatally. Group two consisted of forty Tg2576 and forty-three Wt2576 mice raised on three separate types of drinking

water: zinc enhanced (n = 21), zinc plus copper enhanced (n = 20), iron enhanced (n = 21), and lab tap water (n = 21) beginning during adolescence (Fig. 3).

Group 1	Group 2
Initial exposure prenatally	Initial exposure at adolescence
Tg, n=16	Tg, n=40
Wt, n=18	Wt, n=43
Iron, n=16	Iron, n=21
Lab n=18	Lab, n=21
	Zinc, n=21
	Zinc+Copper, n=20

Figure 3: Design of Experimental Animals

Mice were randomly assigned to one of the water (treatment) groups, with access to food and water ad libitum. Animals were grouped housed, four per cage, in a mouse colony and were handled daily. Animals were tested using the novel object recognition (NOR) task between 12 and 13 months of age. 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 Guidelines.

Water Preparation

Water was prepared in the Biopsychology laboratory at George Mason University. Zinc-enhanced water, 10ppm $ZnCO_3$, was prepared with the addition of a Zinc standard (Zinc, %5 HNO_3) to laboratory tap water (David King Hall, George Mason University, VA). Zinc plus copper-enhanced water, 10ppm $ZnCO_3$ with 2ppm Cu, was prepared with the addition of a zinc standard (Zinc, %5 HNO_3) and a copper standard (Copper, %5 HNO_3) to laboratory tap water. Iron enhanced water, 10ppm $FeNO_3$, was prepared with the addition of an iron standard (Iron, %5HCl) to laboratory tap water. Zinc, copper, and iron metal standards were purchased from SPEX CertiPrep Group, Metuchen, NJ, USA. The pH of the enhanced metal water was tested upon preparation to ensure proper pH for dietary consumption.

Behavioral Testing

NOR

The novel object recognition (NOR) task was performed using animals between the ages of 12 and 13 months of age for both groups one and two. The NOR box is a white four-sided plexi-glass square box that is 18 inches by 18 inches and 9.5 inches in height. A plexi-glass insert is placed on the bottom of the box to which the objects are attached. Inserts for brown and black mice are white while inserts for white mice are black, this is necessary to create the greatest contrast for the tracking computer system (Clever Sys., Inc., Reston, VA). Velcro is used to secure objects to the inserts in the bottom of the NOR box. Velcro was placed on the bottom of the object and in the box: 8 and ¾ inches from two parallel sides of the box and 4.5 inches away from the opposite

parallel sides of the box so that there is 8.5 inches between the two pieces of Velcro. The objects used in this experiment were a small light bulb (upside down), a miniature trophy, a Lego structure, and a baby block with an Easter egg glued on top (Fig. 4).

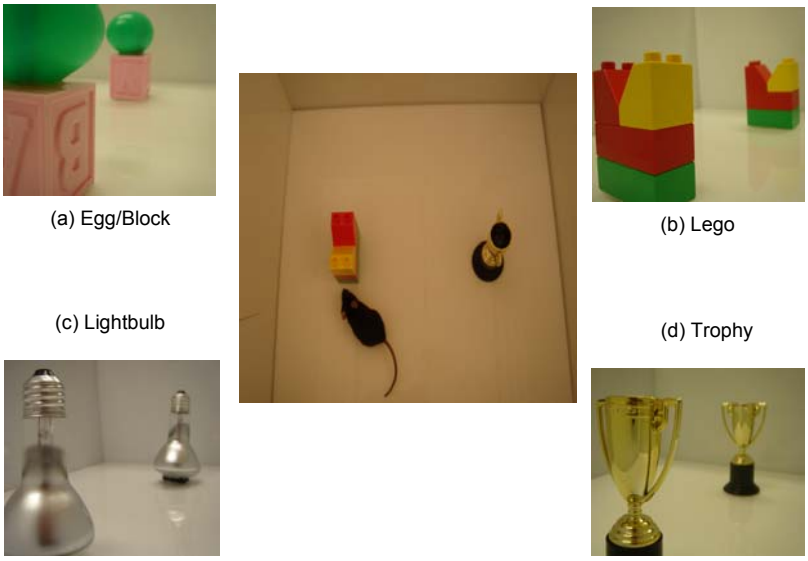


Figure 4: NOR box set-up with a standard and a novel object. Including sample objects of egg/block, lego, lightbulb, and trophy.

The experiment took place in a dimly-lit room with three sets of lights facing the wall and away from the NOR box. A light meter was used to determine that all portions of the box were equally lit and that there were 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 (Fig. 5).

(a) (b) (c)

Figure 5: Novel Object Recognition (NOR) testing room lay-out. (A-B) The room used low-lighting provided by painters' lights at several locations in the room. In addition, the NOR box was set into a larger box, which was covered in black cloth, to prevent mice from jumping directly out of the testing box. (C) Objects were attached to the box using Velcro.

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 is measured through nose sniffing, when the animal points its nose directly toward the

object. Animals were taken in their home cages to a room next to the testing room and allowed to habituate for 10 minutes in the room.

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 have no remaining 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, again 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 middle of the box. Following habituation, there were 3 days of testing the animals for novel object discrimination. During each trial there were 2 objects placed in the box and the animals were in the box for a 5 minute duration. Varying durations of time spent in the box were used to prevent the animal from habituating to a specific time duration spent in the testing box.

The first day post-habituation, an animal was placed in the box for the first time with two of the same object; this object was their standard (object). The standard was present in all other trials for the animal and was tested against a novel object. Following the initial exposure trial, the animal went through three more trials after delays of 1 hour, 24 hours, and 72 hours. One hour 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 has never seen before. Between the initial trial and the 1 hour trial, the

animal was placed back in its cage in the room where it habituated and not back to the colony room. Twenty-four hours later, the animal was again placed in the box with its standard and a second novel object. Seventy-two hours later, the animal was placed in the box with its standard and a third novel object.

Counterbalancing was done for water type, genetic type, object, and object location to ensure no confound due to these extraneous variables (Fig. 6).

Group 1 and Group 2

Trial 1		Trial 2 (1 hr)		Trial 3 (24 hrs)		Trial 4 (72 hrs)	
Block/Egg	Block/Egg	Block/Egg	Lego	Trophy	Block/Egg	Block/Egg	Lightbulb
Lego	Lego	Trophy	Lego	Lego	Lightbulb	Block/Egg	Lego
Trophy	Trophy	Trophy	Lightbulb	Block/Egg	Trophy	Trophy	Lego
Lightbulb	Lightbulb	Block/Egg	Lightbulb	Lightbulb	Lego	Trophy	Lightbulb
Block/Egg	Block/Egg	Lego	Block/Egg	Block/Egg	Trophy	Lightbulb	Block/Egg
Lego	Lego	Lego	Trophy	Lightbulb	Lego	Lego	Block/Egg
Trophy	Trophy	Lightbulb	Trophy	Trophy	Block/Egg	Lego	Trophy
Lightbulb	Lightbulb	Lightbulb	Block/Egg	Lego	Lightbulb	Lightbulb	Trophy

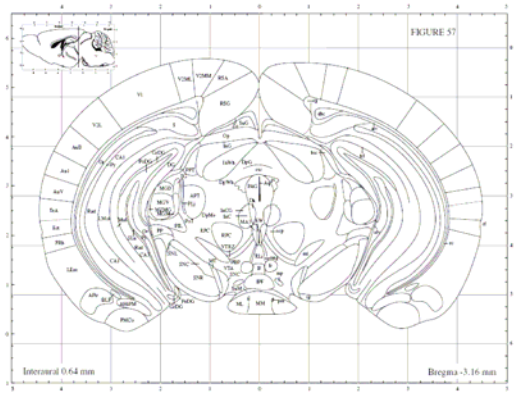
Figure 6: Counterbalance chart for group 1 and group 2. The objects under Trial 1 represent the standard for the animal following that counterbalance pattern. In the following trials (2-4) the two columns under each trial cell represent the left and right positions of the objects. As can be seen, the novel and standard objects switch locations each trial.

Object location was rotated/counterbalanced throughout the experiment. The first time the animal encountered the standard object during the initial trial it was in one location, during the second encounter with the standard (or the 1 hour trial) the object was in the opposite location, during the third encounter (24 hour trial) the standard returned the initial position, as so on. (For example, if standard is first located in the left position for the initial trial, it will be located in the right position for the 1 hour trial, the left position for the 24 hour trial, and the right position for the 72 hour trial.) Object rotation was planned to minimize results due to side preference or memory based on object location versus object identification. This protocol was followed for both groups one (mice raised on lab tap water or iron enhanced water) and two (mice raised on lab tap water, zinc enhanced water, or zinc plus copper enhanced water). An additional delay of 1 week was added for group two where one week later, the animal was placed in the box with its standard object and a fourth novel object.

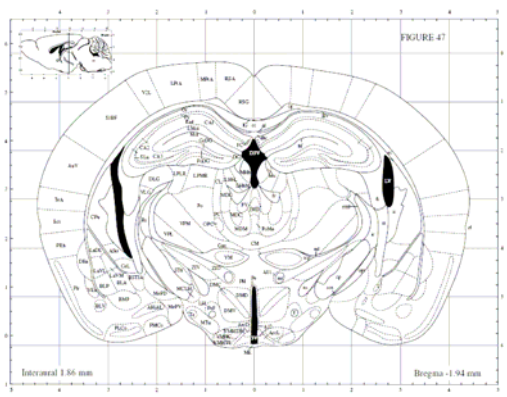
Histological Analysis

After behavioral testing was complete, histological analysis was completed on the brains of mice from both comparison groups. Mice were decapitated using a guillotine in a room different than their home colony room. After the brains were extracted, they were flash-frozen for several minutes in dry ice, then stored in a -80°C freezer for longer term storage. Brains were sectioned with a cryostat in either 10μm or 20μm coronal sections through the hippocampus, surrounding cortex, and basal ganglia on normal glass slides for the following histological analyses. During sectioning the atlas of Pexinos and Franklin (2001) was used for consistency of region of interest (ROI) location. The five

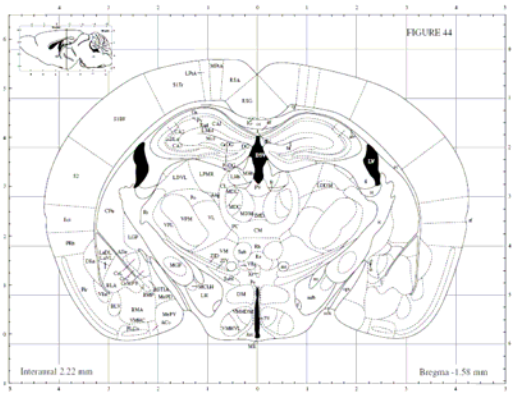
predetermined ROIs are as follows ventral hippocampus (vHC), dorsal hippocampus (dHC), anterior hippocampus (aHC), basal ganglia 1 (BG1), and basal ganglia 2 (BG2) (Fig. 7).



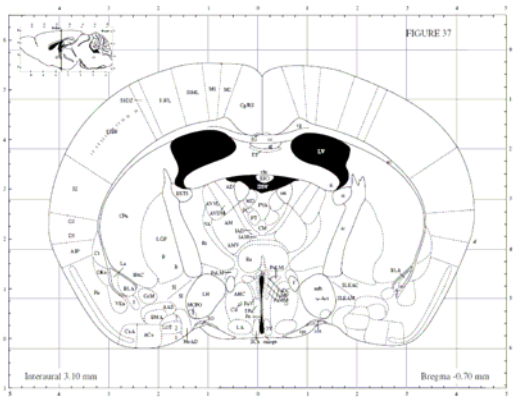
(a) ROI 1: vHC



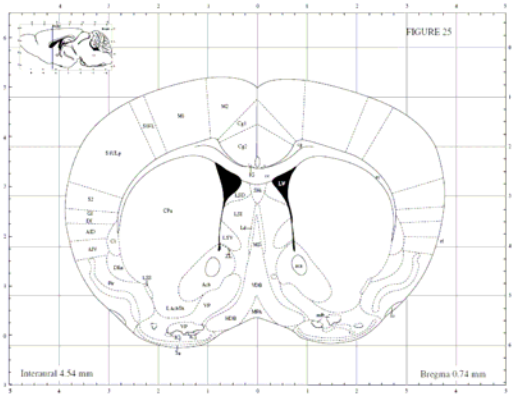
(b) ROI 2: dHC



(c) ROI 3: aHC



(d) ROI 4: BG1



(e) ROI 5: BG2

Figure 7: Regions of Interest (ROI 1-5) which were used for sectioning guidance as taken from Paxinos and Franklin (2001).

Sections were stained with Congo red stain to determine if there are amyloid deposits and plaque levels in the tissue sections. The protocol which was followed 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, coverslip.

After staining, amyloid should stain 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. Images of the slices/amyloid were analyzed using ImageJ, a public domain, Java-based image processing program developed at the NIH (National Institutes of Health, 2007). 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. Additional sections were taken for further analysis at Brookhaven National Laboratory (BNL) to measure metal content and amyloid conformation. Further sections were also taken and may be stained with Prussian blue and Thioflavin to be examined for further plaque and metal content.

Data Analysis

Behavior

Analysis of all behavioral data was completed using the general linear model (GLM). The factors of this GLM were water type, genotype, object, and recognition trial. Statistical data analyses were conducted using SPSS 14.0 statistical software. NOR data was analyzed with the dependent variable as the amount of time spent sniffing (nose sniffing directly towards object) an object. The within group factors of this measure were the objects (novel and standard) and the recognition trials at delays of 30 minutes, 1 hour, 24 hours, 72 hours, and 1 week. Recognition trial used depended on the analysis being conducted and from which group the data were derived. The between group factors were the three water types of zinc, zinc plus copper, iron, and lab tap water, and the two genotypes, Tg and Wt. An outlier analysis was conducted to identify extreme cases, which was defined as $\pm$ 3.0 standard deviation from the mean. Outliers were eliminated only when identified as an outlier in each of the three recognition trials.

The components of the model for the second aim of the study were iron and lab tap water types, Tg and Wt genetic types, novel and standard objects, and retention intervals of 1 hour, 24 hours, and 72 hours. The components of the model for the third aim were zinc, zinc plus copper, and lab tap water for water type, Tg and Wt for genetic type, novel and standard for object, and 30 minutes, 1 hour, 24 hours, 72hours, and 1week for recognition trial.

Included in the statistics of the results section is the effect size as represented by Cohen's d. The effect size is a measure of the strength of a relationship between two variables in a statistical population. In addition to knowing whether a relationship of variables is statistically significant, this measure helps define the size of the relationship. For example, if a relationship is statistically significant, it may be less interesting if the effect size is small. The equation for Cohen's d is: $d = \frac{\bar{x}_1 - \bar{x}_2}{s}$, where the difference of two means is divided by the pooled standard deviation for the data. The equation for pooled standard deviation is: $s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2}}$.

Histology

Images of the Congo red stained sections were analyzed for plaque number and plaque burden. A plaque can be characterized as a small (25μm diameter), medium (50μm diameter), or large (100μm diameter). Hippocampus, cerebral cortex, and basal ganglia were measured for the percent area loaded with plaques (comparing the amount of brain tissue which is burdened with plaques or has no plaques). The Image J program can help with the calculation analysis, but it does remain a hand scoring program where statistics are obtained though user-defined selections, or hand drawn scoring areas. Thus, in an effort to produce the most reliable results, several researchers were involved in the scoring of each series of data. The following figure shows which researchers were involved with the scoring of each set of data (Fig. 8); there was high inter-rater reliability.

Group	Water group	Researcher
Group 1	iron, lab water	CG, JT
Group 2	iron, lab water	AB, EG, CG
Group 2	zinc, zinc plus copper	EG, CG

Figure 8: Researchers involved with the scoring of plaque data from the brain tissue of animals who consumed various metal enhanced water.

An analysis using the GLM was performed to compare plaque load (plaque load = total plaque area) with the genotype and water type across brain regions. Genotype and water type were the same parameters as discussed. Brain regions, or ROIs, were defined as ventral hippocampus (vHC), dorsal hippocampus (dHC), anterior hippocampus (aHC), basal ganglia 1 (BG1), and basal ganglia 2 (BG2) (Fig. 7).

CHAPTER 3: RESULTS

Hypothesis Statement

Due to the complexity of this study and the groups, the hypotheses of this study are not discussed in a simple, direct order (i.e., results of hypothesis one, results of hypothesis two, etc.). Therefore, a figure has been included in this section (Fig. 9) to specify each hypothesis, as well as to indicate where each hypothesis is discussed in this chapter.

	Hypothesis	*Learning and Memory Ability Impairment*
Group 1	*H1*	Tg > Wt
	H2a *H2b*	Prenatal Fe > LW Fe Tg > Fe Wt ≥ LW Tg > LW Wt
Group 2	*H1*	Tg > Wt
	H3a *H3b* *H4a* *H4b*	Zn > LW Zn Tg > Zn Wt ≥ LW Tg > LW Wt Zn > ZnCu > LW Zn Tg > Zn Wt ≥ ZnCu Tg > ZnCu Wt ≥ LW Tg > LW Wt
Group 1 vs Group 2	*H2a* *H2b*	Preadolescent Fe > Adolescent Fe > LW Fe Tg > Fe Wt ≥ LW Tg > LW Wt

Abbreviations: Fe = Iron; Zn = Zinc; ZnCu = Zinc plus Copper; LW = Lab Water; Wt = Wild-type ; Tg = Transgenic

Figure 9: Hypothesis Summary Across Groups

Experimental Animal Group Statement

The animal members of the treatment groups and analysis groups are equally complex. An additional figure has been included in this section (Fig. 10) to clarify the treatment and analysis groups. Below is a brief description of the animals which comprise each analysis group in this chapter.

Group 1: Animals raised on iron-enhanced water or lab tap water with initial prenatal exposure.

Group 2: Animals raised on iron-enhanced water, zinc-enhanced water, zinc plus copper-enhanced water, or lab tap water with initial exposure beginning during adolescence.

Group 1 versus Group 2: A comparison of animals raised on iron-enhanced water with initial exposure either beginning prenatally or beginning during adolescence.

Group 1	Group 2
Initial exposure prenatally	Initial exposure at adolescence
Tg, n=16	Tg, n=40
Wt, n=18	Wt, n=43
Iron, n=16	Iron, n=21
Lab n=18	Lab, n=21
	Zinc, n=21
	Zinc+Copper, n=20

Figure 10: Design of Experimental Animals

Novel Object Recognition (NOR)

Group 1 (raised on iron-enhanced water or lab tap water)

The main finding of these data was that transgenic mice showed less novel object recognition than wild-type mice. A 2 x 2 (object x genotype) repeated measures ANOVA showed that the novel object was significantly preferred over the standard object as measured by sniffing duration ($F[1,60] = 8.58$, $p < .01$, $d = .22$) for transgenic and wild-type animals analyzed together. There was a significant interaction of genotype and object ($F[1,60] = 4.97$, $p = .034$, $d = .14$). Transgenic mice sniffed for less time on the novel objects than the wild-type animals (Fig. 11), supporting hypothesis one that transgenic animals would be more impaired than wild-type animals in the NOR task. In the transgenic animals compared to the wild-type animals, there was also significantly less overall sniffing on both the standard and novel object ($F[1,30] = 6.51$, $p = .016$, $d = .18$; Fig. 12). This reduction can be seen in the lower two sets of points on the figure (Fig. 12) where transgenic iron and lab animals have the lowest sniffing levels on both the standard and novel objects.

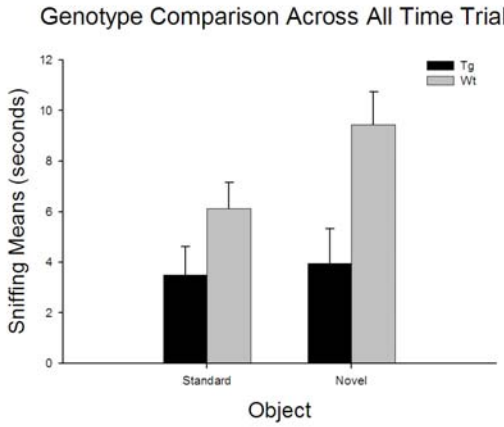


Figure 11: Sniffing duration means by genotype on standard and novel objects for Group 1. Data points represent group means ± the standard error of the mean.

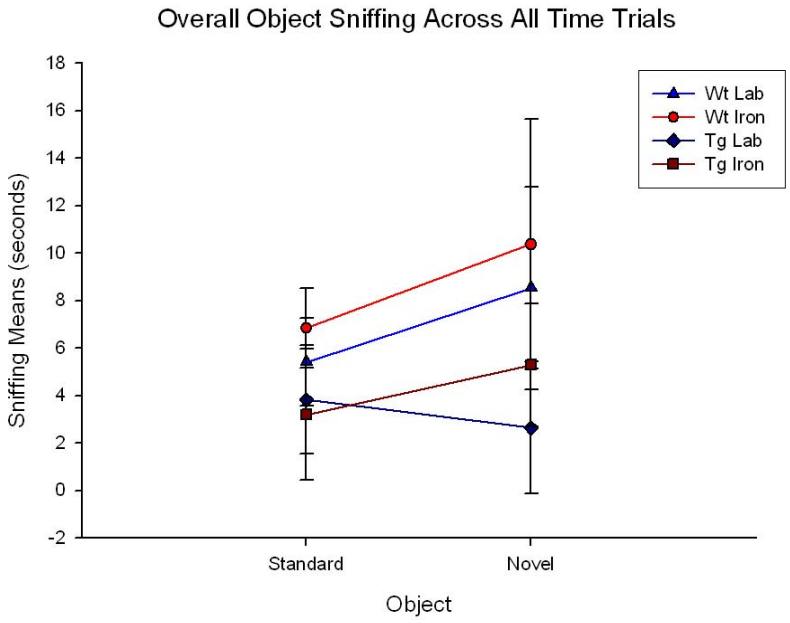


Figure 12: Mean sniffing duration on standard and novel objects for Group 1. Data points represent group means ± the standard error of the mean.

There was no main effect of water type as analyzed by a 2 x 4 x 2 x 2 (object x retention interval/trial x water type x genotype) repeated measures ANOVA which showed there were no significant sniffing differences detected across lab or iron water types (F[2,28] = 0.83, p = .44, d = .06). As indicated by a one-way ANOVA, for the 1 hour data, representative of a shorter retention interval for object recognition, the only significant effect between genotypes was found (F[1,30] = 4.19, p < .05; Fig. 13). This is reflected in the lower two points that occurred at 1 hour, where the difference for sniffing time on the standard compared to the novel was significantly lower in the transgenic lab and iron animals than the wild-type lab and iron animals. There was not a significant interaction between genotype and water type (F[1,30] = .04, p = .84, d = .001).

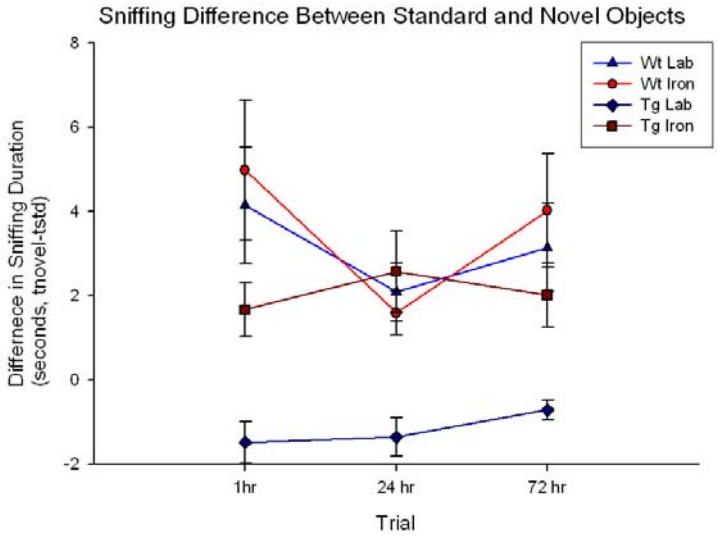


Figure 13: Difference scores for sniffing duration on the novel and standard objects for Group 1. Data points represent group means ± the standard error of the mean.

Both wild-type (lab and iron) groups and the transgenic iron group, with the exception of the transgenic lab group, sniffed the novel object for a longer duration than the standard, exhibiting novel object recognition (Fig. 13). Different results for the iron and lab water transgenic groups could indicate an enhancement of novel object recognition due to the effect of iron in the transgenic animals. In the wild-type group, the iron animals also sniffed more on the novel object versus the standard. All groups appear to be similar except for the transgenic lab water animals (Fig. 13). The iron animals sniffed more than the lab animals, regardless of genotype, on either the standard or the novel object (Fig. 14).

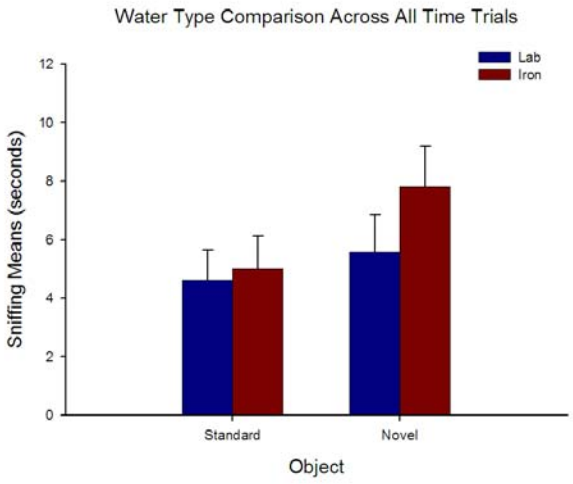


Figure 14: Sniffing duration means for water type on standard and novel objects for Group 1. Data points represent group means $\pm$ the standard error of the mean.

Group 2 (raised on iron-enhanced water, zinc-enhanced water, zinc plus copper-enhanced water, or lab tap water)

The main finding of these data was that transgenic mice showed less novel object recognition than wild-type mice. Overall, all animals sniffed for a longer duration on the novel object (9.69 $\pm$ 0.50) than on the standard object (8.16 $\pm$ 0.58) exhibiting novel object recognition (Fig. 15). A 2 x 2 (object x genotype) repeated measures ANOVA showed that there was a significant interaction of object and genotype (F[1,73] = 4.78, p = .03, d = .06) in that transgenic mice sniffed significantly less on the novel object (Figs. 15B, 15C). All wild-type animals followed this pattern of sniffing more on the novel object, but only transgenic iron animals followed the pattern. This interaction indicated that wild-type mice (11.32 $\pm$ 0.70) sniffed longer on the novel objects than the transgenic animals (8.05 $\pm$ 0.72) (Fig. 15), supporting hypothesis one that transgenic animals would be more impaired than wild-type animals in the NOR task. However, there was not a significant interaction of genotype, object, and water type (F[3,73] = 0.19, p = .90, d = .008), indicating that there was no interaction of these three variables.

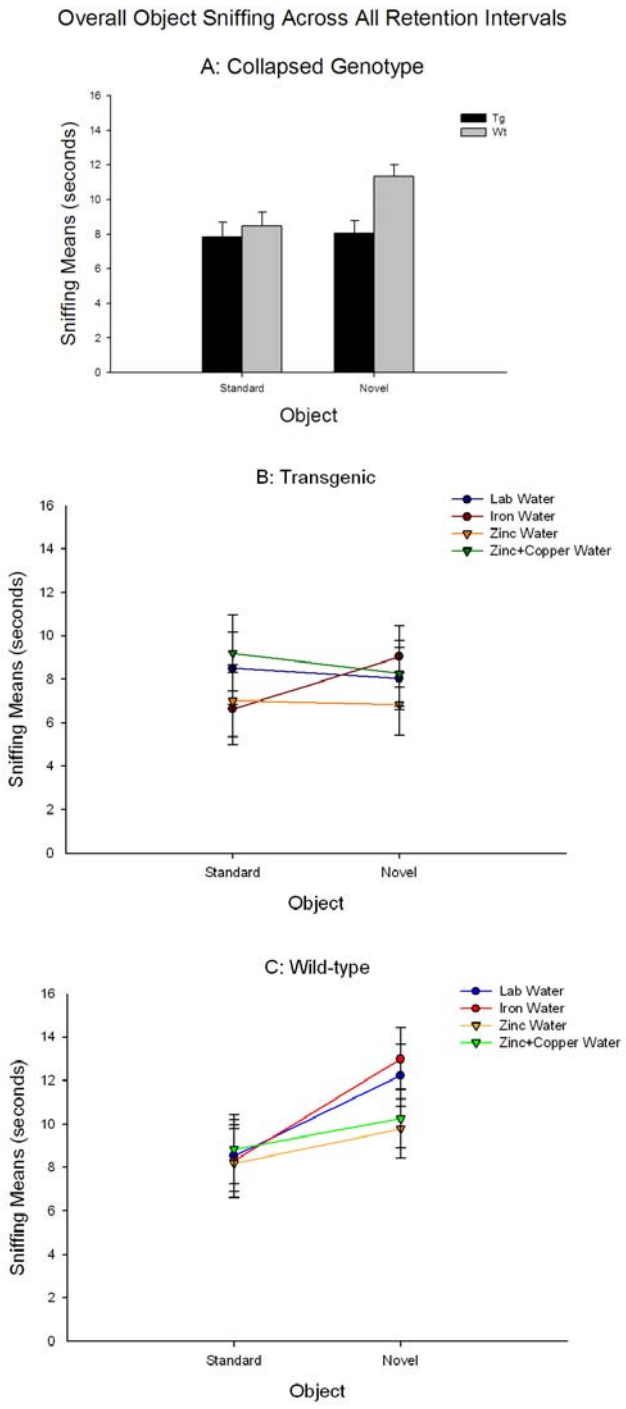


Figure 15: Overall sniffing duration on the standard and novel object for (a) transgenic animals and (b) wild-type animals and (c) by collapsed genotype for Group 2. Data points represent group means $\pm$ the standard error of the mean.

These (group 2) data show the same pattern as was seen with the first group of animals (group 1: iron and lab water). Wild-type mice sniffed significantly more on the novel object than the standard, which is to be expected. But what is unexpected is that, while transgenic mice raised on lab water fail to show novel object recognition, those transgenic mice on iron still maintain novel object recognition (exhibited through greater duration sniffing on the novel object; Fig. 15B).

A 2 x 5 x 4 x 2 (object x retention interval/trial x water type x genotype) repeated measures ANOVA showed there were no overall significant sniffing differences detected across water types (F[9,219] = 0.928, p = .50, d = .04). There was a main effect of genotype across trials (F[1,73] = 4.60, p = .03, d = .06; Fig. 16). In the transgenic animals, there was a trend for decreased novel object recognition compared to standard object recognition (F[1,73] = 3.56, p = .06, d = .05) across all trials (Fig. 16). There were no significant differences detected between any of the four water types (F[3,73] = 0.52, p = .67, d = .02) and no significant main effect of water type across trials (F[9,219] = 1.02, p = .43, d = .04) (Fig. 16). These results do not support hypotheses three or four, that zinc would have a negative affect on object recognition and that copper would enhance the object recognition compare to that decreased by zinc although still showing decreased object recognition.

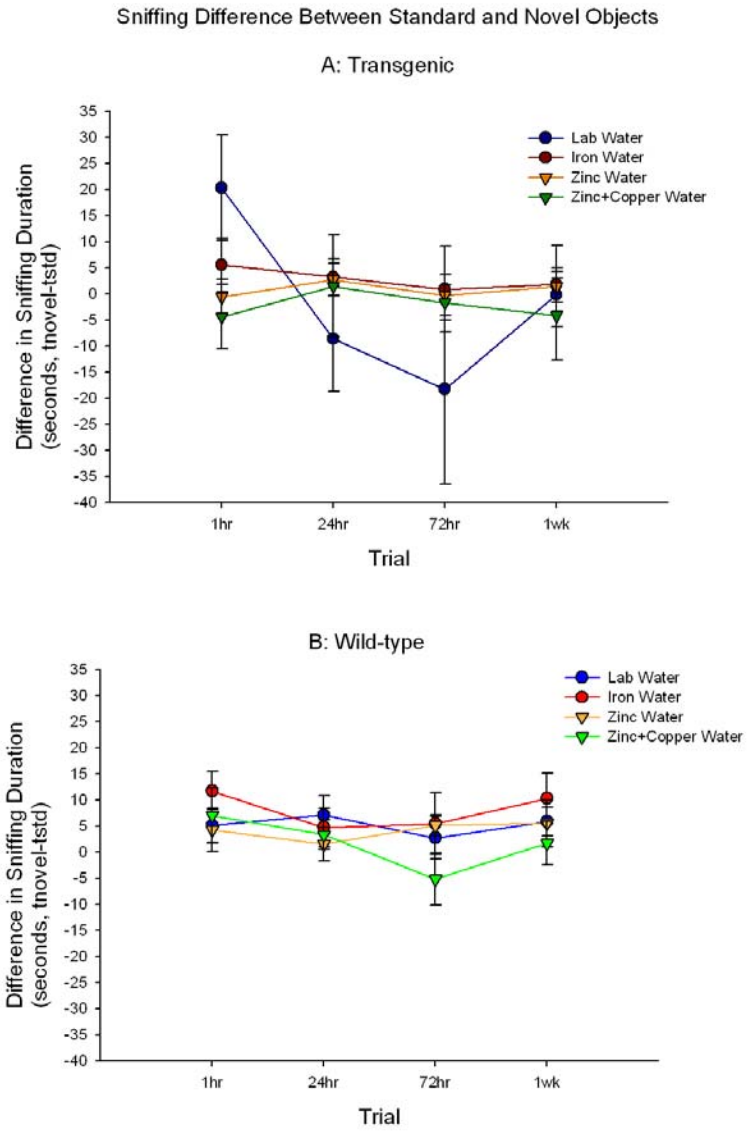


Figure 16: Difference scores for sniffing duration on the novel and standard objects for (a) transgenic animals and (b) wild-type animals for Group 2.
Data points represent group means $\pm$ the standard error of the mean.

For zinc and lab animals retested at 30 minutes, after original testing trials of 1 hour through 1 week, there was no significant effect of water type across trials (F[4,144] = 1.55, p = .19) and no significant main effect of genotype across trials. Transgenic and wild-type zinc animals showed strong sniffing duration at 30 minutes, but this diminishes by 1 hour (Figs. 17, 18). This indicates that there is a change in sniffing behavior at 1 hour.

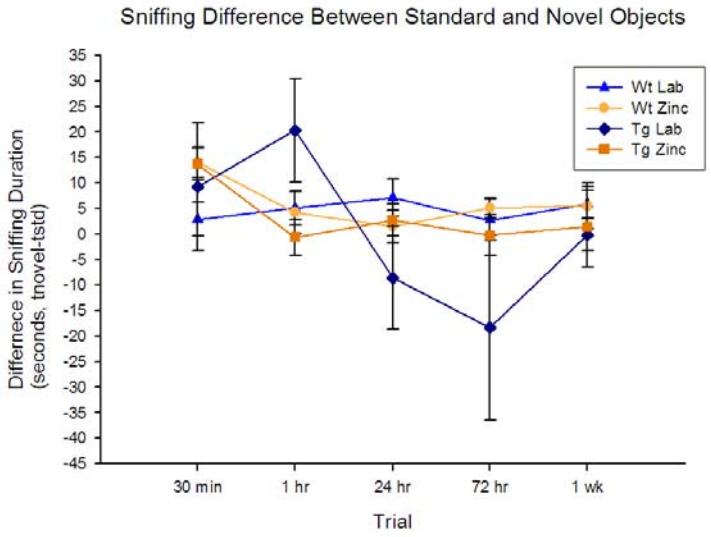


Figure 17: Difference scores for sniffing duration on the novel and standard objects for zinc animals in Group 2. Although run separately, the 30 minute retention interval is displayed as the first interval to represent the shortest retention time.
Data points represent group means ± the standard error of the mean.

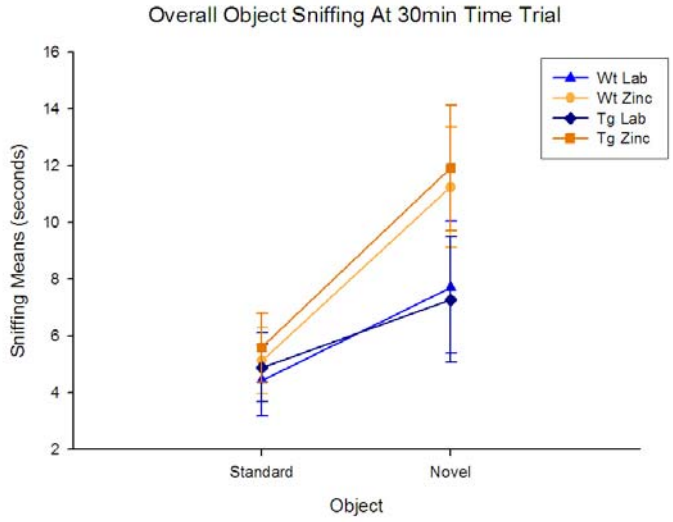


Figure 18: Overall sniffing duration on the standard and novel object only at the 30 minute interval for Group 2. Data points represent group means ± the standard error of the mean.

Group 1 versus Group 2 (comparison of animals raised on iron-enhanced water or lab tap water from different ages)

To determine the difference of novel object recognition between animals raised on iron-enhanced water beginning prenatally versus those raised on iron-enhanced water beginning during adolescence, a repeated measures ANOVA was run. Animals raised on iron-enhanced water beginning prenatally sniffed less overall than those raised on iron-enhanced water beginning during adolescence (Table 1). There was no significant difference in novel object recognition as assessed by sniffing duration for any of the trials between the two cohorts, shown by a 2 x 3 (group x retention interval) repeated measures

ANOVA (F[2,71] = 1.47, p = .24, d = .04). These results do not support hypothesis two, that prenatal exposure to iron would have a more negative affect on object recognition than initial exposure to iron during adolescence.

Table 1: Overall sniffing duration differences on objects for iron and lab water animals in Group 1 and Group 2 across time trials.
Data represent group means ± the standard error of the mean.

	1 hour	**24 hours**	**72 hours**
Group 1	3.46 ± 1.90	2.54 ± 1.73	1.64 ± 1.70
Group 2	11.01 ± 3.13	1.60 ± 3.01	2.31 ± 5.30

Histology, Congo Red

Group 1 (raised on iron-enhanced water or lab tap water)

Sectioned brain tissue of transgenic mice, stained with Congo red and viewed under a light microscope, appear with red to pink amyloid deposits in various regions of the brain depending on the progression of the disease and water type of the animal. Amyloid plaques present on brain sections viewed under a fluorescent/polarizing microscope should birefringe and appear apple green in color if the plaques include β-pleated sheet conformation amyloid. This was the case in these experimental animals (Fig. 19).

Analysis was performed on the red stained plaques, while the green color indicative of β-pleated sheet amyloid was used to separate plaques from artifacts.

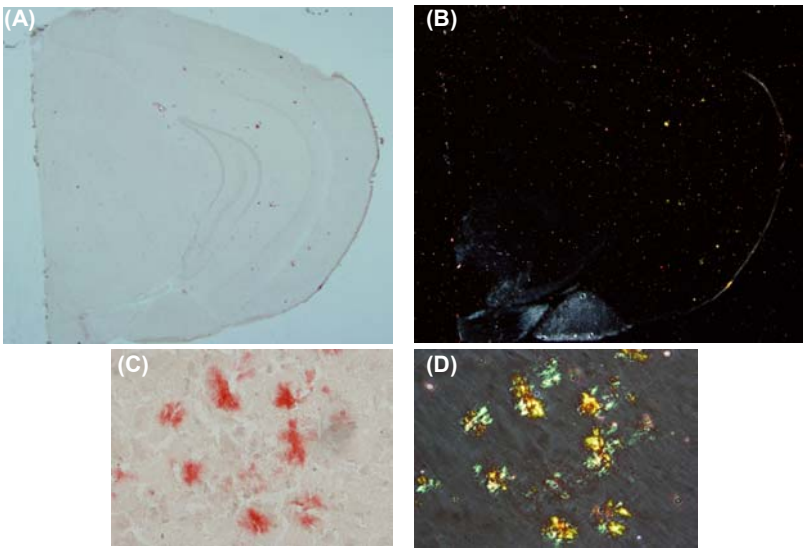


Figure 19: Congo Red stained tissue, from a group 1 transgenic animal on iron water, without (A,B) or with (C,D) polarization. Whole left hemisphere (A,C) and individual plaques (B,D) for Group 1.

Results of a 5 x 2 (ROI x water type) ANOVA showed a trend for plaque load (plaque load = total plaque area) differences across water type, with lower plaque levels in mice raised on iron enhanced water ($F[1,13] = 3.92$, $p = .07$; Fig. 20). Plaque load levels were also dependent on region of interest in the brain, across both iron and lab tab water ($F[4,10] = 3.80$, $p < .05$; Fig. 20) with higher plaque burden in the hippocampus than the basal ganglia. This analysis included the three combined hippocampal regions (0.02 $\pm$ 0.007, in mm^2) and the two combined basal ganglia regions (0.01 $\pm$ 0.006, in mm^2). However, the highest levels were seen in the posterior basal ganglia of the lab water group.

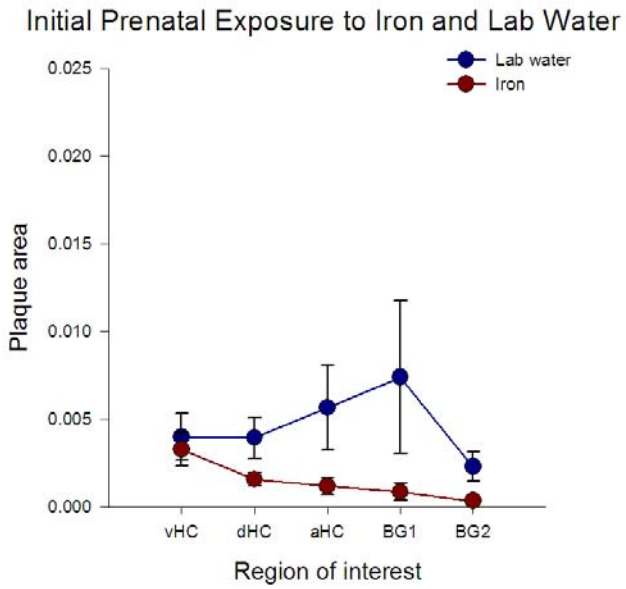


Figure 20: Overall Plaque load analysis across region of interest for Group 1. Data points represent group means $\pm$ the standard error of the mean, in mm^2. (For description of ROIs, see Fig. 7 in Methods: Histological Analysis)

Group 2 (raised on iron-enhanced water, zinc-enhanced water, zinc plus copper-enhanced water, or lab tap water)

Sectioned brain tissue of transgenic mice, stained with Congo red and viewed under a light microscope, appear with red to pink amyloid deposits in various regions of the brain depending on the progression of the disease and water type of the animal. Amyloid plaques present on brain sections viewed under a fluorescent/polarizing microscope should birefringe and appear apple green in color if the plaques include β-pleated sheet conformation amyloid. This was the case in these experimental animals (Fig. 21). Analysis was performed on the red stained plaques, while the green color indicative of β-pleated sheet amyloid was used to separate plaques from artifacts.

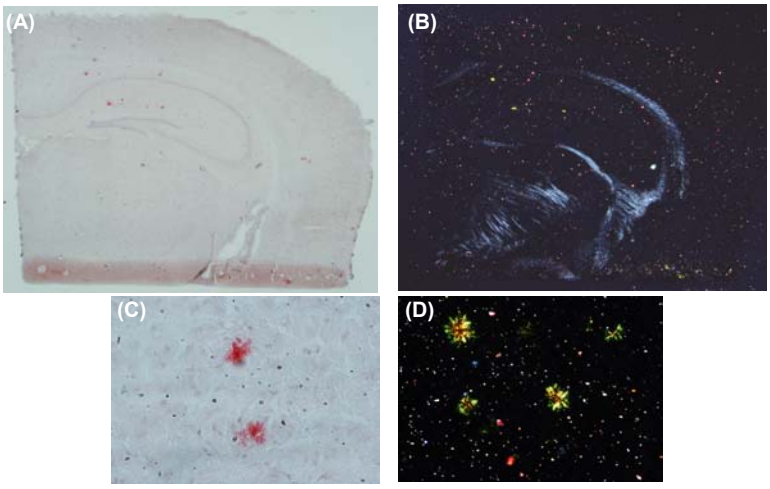


Figure 21: Congo Red stained tissue, from a group 2 transgenic animal on iron water, without (A,B) or with (C,D) polarization. Whole left hemisphere (A,C) and individual plaques (B,D) for Group 2.

There was a significant difference of plaque load levels dependent on region of interest across both iron and lab tap water (F[4,16] = 22.09, p < .001; Fig. 22). When comparing mice raised on iron enhanced water compared to mice raised on lap tap water, using a 5 x 2 (ROI x water type) ANOVA, there was no overall significance for plaque load differences across water type (F[1,40] = 2.41, p = .15). An exception was a significant difference between the mice raised on iron enhanced water and lab tap water in the 5th ROI (basal ganglia 2) (F[1,40] = 11.68, p = .001) with a lower plaque load in the iron group (Iron: 0.004 $\pm$ 0.0006; Lab: 0.007 $\pm$ 0.0009, in mm^2) (Fig. 22).

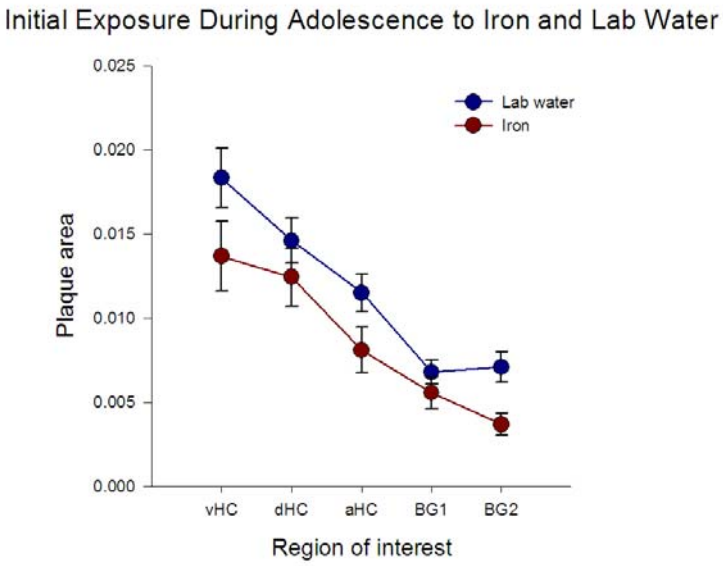


Figure 22: Overall Plaque load analysis across region of interest for Group 2, iron and lab water animals only. Data points represent group means $\pm$ the standard error of the mean, in mm^2. (For description of ROIs, see Fig. 7 in Methods: Histological Analysis)

When comparing mice across all four water groups, using a 5 x 4 (ROI x water type) ANOVA, there was no significant difference for plaque load differences across water type (F[1,35] = 0.70, p = .56; Fig. 23A). However, there was a significant difference of plaque load levels dependent on region of interest across all four water types (F[4,140] = 29.50, p < .001; Fig. 23B), with higher plaque burden in the hippocampus than the basal ganglia. This included the three combined hippocampal regions (0.08 ± 0.009, in mm^2)) and the two combined basal ganglia regions (0.02 ± 0.003, in mm^2). The highest levels were seen in the ventral hippocampus of both the iron and lab water groups.

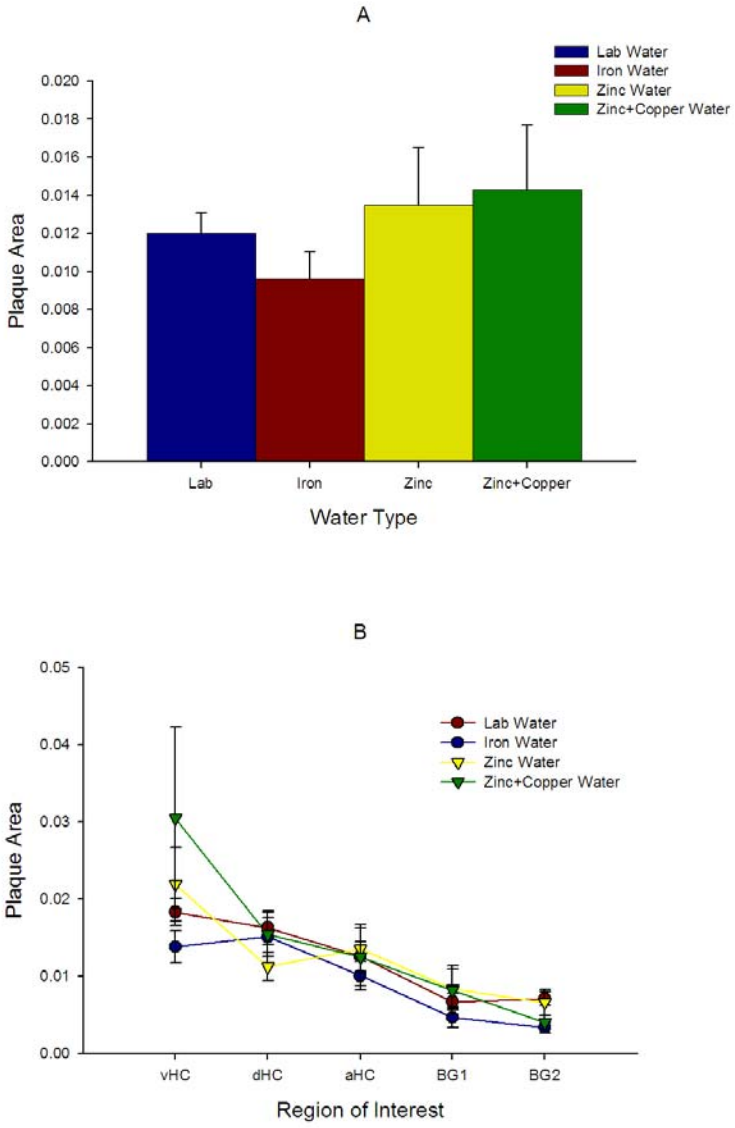


Figure 23: Overall plaque load analysis across (a) water type for all regions and (b) region of interest for Group 2. Data points represent group means $\pm$ the standard error of the mean.

A large number of statistical tests were conducted for the analysis of this project, which could have been sufficient for the running of a Bonferroni correction. However, it was accepted that this correction did not have to be conducted since there were a large number of groups (metal water groups, transgene groups, etc.) and low sample size numbers within each of these groups.

CHAPTER 4: DISCUSSION

The purpose of this study was two-fold. First, it was important to examine the effect of metals in the drinking water (specifically zinc, copper, and iron) on the disease progression and brain pathology of Alzheimer's disease in an animal model. It was also important to determine if the novel object recognition behavioral task was a candidate to become a standard recognition memory task in transgenic mice.

Transgenic animals showed decreased novel object recognition compared to wild-type mice. This was expected as the transgenic mice of the AD type should show decreased recognition memory for previously presented information and therefore would be less likely to sniff discriminately on a novel object compared to a previously presented object. This shows that the novel object recognition task has the potential to be used as a standard recognition (memory) deficit task for transgenic mice. The NOR task should therefore serve as an appropriate companion task for the Morris water maze (MWM) which has demonstrated its capacity as a reliable standard spatial memory task in Tg2576 mice (Hisao et al., 1996).

There was no overall change in novel object recognition due to consumption of metal enhanced water types (iron, zinc, or zinc plus copper enhanced water). However, across water groups, there was an effect of genotype. Interestingly, there does appear to be evidence that iron enhancement in the water may lead to the enhancement of novel

object recognition in both wild-type (Wt) and transgenic (Tg) mice. As chelation therapy is generally used to remove metals from the brain, including iron (House et al., 2004; Shin et al., 2003), this is a very interesting finding that must be further researched.

In the transgenic animals of group 1, in which animals received lab water or iron-enhanced water beginning prenatally, the lab water animals showed less sniffing on the novel compared to the standard while the iron water animals showed more sniffing on the novel compared to the standard. This is in contrast to the wild-type animals of these two water groups where the wild-type animals of the lab water and iron water animals both showed more sniffing on the novel compared to the standard. This indicates that the transgenic lab water animals may suffer from a recognition memory deficit due to their genetic mutation of the human amyloid precursor protein (hAPP) (Hisao et al., 1996; Hisao Ashe, 2001), while the transgenic iron water animals may exhibit a rescue of this recognition deficit due to the iron water consumption. Transgenic animals of the second group, with exposure to metal enhancement beginning during adolescence, also showed the same pattern of decreased sniffing levels on the standard object compared to the novel object for the lab water animals, but more sniffing for the iron water animals. Regardless of the exposure time to iron-enhanced water, there is evidence to suggest a remediation effect of recognition memory for the transgenic APP2576 mice due to iron enhancement. It is important to note that the data had high variance and there were small effect sizes.

Unexpectedly, there was no difference in novel object recognition between animals that had been raised on iron enhanced-water beginning prenatally versus those raised on iron enhance-water beginning during adolescence. It was predicted that object

recognition would be even more impaired in animals which had received extended exposure to iron (prenatal exposure animals) compared to those which had received less exposure. Following the behavioral findings that were seen in these results (e.g., enhanced novel object recognition due to iron supplementation), a new expectation might have been that the animals that received the most exposure to iron would perform the best on the NOR task. Interestingly, the animals exposed to iron beginning prenatally did show lower averages of sniffing as compared to animals exposed to iron during adolescence. Although increased exposure to iron did not impair novel object recognition, it did diminish the level of sniffing. These findings suggest that iron may affect the brain differently than was expected; iron may be negatively affecting a different region of the brain than was expected.

In transgenic animals exposed to zinc enhanced water there was more recognition of the novel object than the standard object at 30 minutes. These findings indicate that there is no negative effect due to zinc. As zinc animals performed better in the NOR task, this gives the appearance that zinc may have been beneficial to object recognition. The addition of copper did not lead to remediation as was expected. Conversely, in transgenic animals exposed to zinc plus copper enhanced water, the same pattern was exhibited as for lab water transgenic animals; there was less recognition of the novel object than the standard object.

Different results were previously reported in our lab in both rats (Railey, Micheli, Wanschura, Flinn, unpublished data) and transgenic Alzheimer's mice (Railey, Smith, Micheli, Morgan, Fitzgerald, VeeJay, Flinn, et al., 2006). Although the rats tested were

not transgenic and did not represent the disease model, they showed a change of learning and memory following metal administration. Rats receiving supplementation with zinc plus copper enhanced water exhibited freezing (in fear condition) and latency (in Morris water maze) levels that were much closer to control animals than those exposed to zinc enhanced water alone (Railey et al., unpublished data). The mice used were the same group of animals (group 2) as in the present study and therefore do exhibit the Alzheimer's disease model phenotype. However, when tested using the Morris water maze, a small amount of supplementary copper reduced learning deficits associated with enhanced zinc exposure. Iron also produced impairments in the spatial Morris water maze (Railey et al., 2006). The differences in these findings and contrasting results may be explained due to the differing type of tasks and corresponding brain regions required for these tasks and affected by these metals. Although both hippocampus-dependent tasks, novel object recognition is a non-spatial, recognition memory task while Morris water maze is a spatial task. These two tasks may require different areas of the hippocampus for task completion, or as has recently been suggested, spatial memory may require more hippocampal tissue than recognition memory (Broadbent, Squire, Clark, 2004). The entorhinal cortex may be involved and play an essential role in recognition memory, as discussed below.

There could have been a potential confound in this experiment of smell due to the zinc enhancement. Zinc causes an increase of smell and taste acuity (Brandao-Neto, Stefan, Mendonca, Bloise, Valeria, Castro, 1995) in human children and in rats. Due to the nature of the NOR task this enhanced smell acuity could have allowed for the

measurement of the more novel smell. In this instance, the more novel smell may have been the novel object which, regardless of attempts to be cleansed, may have had residual smell remaining from the previous animal. Whereas the standard object would still have the testing animals' smell and therefore would not be novel to that animal. This result would give findings opposite of the valid result and show that the standard object was more interesting that the novel object. It must be noted that there are studies which have found that zinc can decrease smell ability, in one case zinc sulfate irrigation caused axonal degeneration and outward migration of olfactory Schwann cells (Chuah, Tennet, Jacobs, 1995).

The brain data are consistent with the conclusion of the behavioral data that iron remediated the effects of the hAPP transgene in the transgenic animals. In the first group, the iron water animals show a decreased plaque load compared to the lab water animals. In the second group, there was no significant difference between the iron water animals and the lab water animals with the exception of the 5th region of interest, basal ganglia 2 (BG2). However, the lowest overall plaque levels were found in iron water animals with the highest overall plaque levels found in zinc plus copper water animals. As copper is expected to remediate the effects of zinc on plaque accumulation, it was unexpected to find this group with highest plaque accumulation (Diebel, Ehmann, Markesbery, 1996; Railey et al., 2006). However, there has been some literature in agreement with this finding. Lovell et al. (1998) showed an overall increase in zinc, copper, and iron in plaques, and specifically found a significant increase of copper in plaque rims.

In both groups 1 and 2, there was an increased plaque burden in the hippocampus compared to the basal ganglia. This was expected as the entorhinal cortex and the hippocampus are among the first areas of the brain to develop plaques in Alzheimer's disease, with plaque formation developing much later in sensory and motor areas, such as the basal ganglia (Braak & Braak, 1997). Here again a relationship is seen with the behavioral data, as the hippocampus and entorhinal cortices are linked to episodic and semantic knowledge, semantic knowledge playing an important role in the object discrimination tasks (Vargha-Khadem et al., 1997). Interestingly, group 2 had about two to four times the plaque load that group 1 had; There is no obvious explanation for why this would have occurred. There was no significant age difference at the time of sacrifice and the only major difference in the animals was that group 1 animals were bred and raised in our facility while group 2 animals were purchased and delivered to our facility at 3 months. Additionally, group 1 animals started on iron-enhanced water earlier than group 2 animals since they did not arrive at our facility until 3 months of age.

It is important to note that researchers are examining the possibility that the hippocampus is not essential for object recognition and that the entorhinal cortex may play a more essential role in recognition memory tasks. Hippocampal damage does not appear to be sufficient to impair recognition memory (Aggleton and Brown, 1999; Lehmann, Glenn, Mumby, 2007). Therefore, other areas of the brain must also be essential for recognition memory (i.e., prefrontal region for memory retrieval). Current research examines the perirhinal, parahippocampal, and entorhinal cortices as possible areas essential to the recognition memory pathway (Barense, Henson, Lee, Graham,

2009). Future research should examine the effect zinc, copper, and iron pathology on not only the hippocampal and basal ganglia regions, but certain structures within the medial temporal lobe circuit which includes these additional areas (perirhinal, parahippocampal, and entorhinal cortices) which may be essential to recognition memory and object discrimination.

There were some limitations and potential confounds to this study that should be addressed in any future studies relating to the topic of metal-enhancement in the diet or water for the study of the Alzheimer's disease model. First, only one concentration of metal was used for each metal type; varying levels should be used in a follow-up studies. This will allow for the determination of what is a safe enhancement level for specific metals versus a harmful enhancement level, if there is even room at any level to make this distinction. Second, the animals were exposed to the metals in a chronic dosage method over the long-term through the drinking water. It might be of interest to vary the administration route, for example, in the food instead of the water or an acute, mass dose in a tablet form. Variation of administration route would be interesting to help determine if there are differing outcomes due to the administration route. Although these two examples of alternative administration routes are both oral, there is enough difference in the route of administration (i.e., a solid versus a liquid) that there could be important differences such as differential absorption rates of the metals. Due to these limitations, a four parameter logistic model (4PL) analysis might be an appropriate analysis method for a follow-up study. A 4PL analysis will allow for the comparison and identification of statistical differences between resulting dose-response curves.

Third, in the NOR task, the object location was switched after every trial to prevent a side preference. This protocol choice was made from current literature at the time of experimental planning, which suggested that the object location should be switched to counterbalance a location preference (Bredy, Brown, Meaney, 2007; Dix and Aggleton, 1999). There is some debate about whether or not there should be a switching of the object location during a non-spatial NOR task. It is possible this may have instead actually lead to a confound linked to this switching, and there is no way to statistically parse this out to determine the reality. The animal may have expected the novel object to be on the same side as it was in the previous trial, and when it was not, this may have caused the animal to sniff more to be sure it was in fact the object with which they already had experience. Researchers are also using an alternative NOR box apparatus which include more than two objects. This set-up can reduce the likelihood of a location switching preference. This option may make the task different and introduce a spatial component to the NOR task which can allow for the testing changes in different brain regions (i.e., the basal ganglia instead of the hippocampus).

Fourth, there needs to be testing of object recognition at shorter intervals. This paradigm used 1hour, 24 hours, 72 hours, and 1 week. In previous pilot work of this lab, 5 minute and 30 minute paradigms have been utilized (as well as for one group in this study). While 5 minutes is too short, 30 minutes does appear to be a good time for object recognition, while 1 week tends to be too far away, especially in the diseased animal. Therefore, 30 minutes, 1 hour, 24 hour seems like a good possible paradigm for a future study; with the possibility of 15 minutes and 72 hours on either side.

Fifth, as the brain plaque data were hand scored, there was less standardization than there could have been. In future studies, plaques should be characterized using an automated system (e.g., Bioquant Nova Prime, Nashville, TN), which may provide more reliable data. Lastly, both the behavioral and brain conclusions of this study relied on the data collection from only one task (behavioral: NOR; brain: plaque counts). Future studies should extend to additional behavioral tasks and brain measurements to reach conclusions with increased strength and reliability.

Summary

As expected, the transgenic animals sniffed less on a novel object than a standard object, showing decreased novel object recognition. Iron appears to have a rescuing effect on the recognition memory deficit caused by the transgenic mutation given to APP2576 mice, as exhibited through enhanced novel object recognition compared to the lab animals. These results, in contrast to those of the Morris water maze, indicate that metals may affect various regions of the brain in different ways, therefore producing varying cognitive deficits and/or enhancements. The findings of this study show evidence that metals in the diet can affect brain pathology and cognition (as described through an object recognition task) in transgenic mice. With more extensive studies, there is a possibility that iron enhancement could be factored into therapies associated with diagnosed Alzheimer's disease (AD) patients or used as a preventative measure to prevent cognitive decline for those with the genetic predisposition for AD. These future studies need to investigate varying metal concentrations and dosage, length of exposure to metal enhancement, varied memory paradigms, and additional brain analysis.

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CURRICULUM VITAE

Caitlin M. Groeber was born in Indianapolis, Indiana, and is an American citizen. She graduated from Fairfax High School, Fairfax, Virginia in 2001. She received her Bachelor of Science in Biochemistry and Bachelor of Science in Psychology, with a Minor in Chemistry, from Virginia Polytechnic Institute and State University, Blacksburg, Virginia in 2005. She received her Master of Arts in Psychology from George Mason University, Fairfax, Virginia in 2009. She is currently pursuing her Doctor of Philosophy in Psychology at George Mason University.