EFFECTS OF PRENATAL DIETARY ENHANCEMENT OF IRON ON ßETA AMYLOID (Aß) PLAQUES AND BEHAVIOR IN WT2576 AND TG2576 MICE

by

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ABSTRACT

EFFECTS OF PRENATAL DIETARY ENHANCEMENT OF IRON ON ßETA AMYLOID (Aß) PLAQUES AND BEHAVIOR IN TG2576 AND WT2576 MICE

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This thesis investigated the relationship of iron in the brain enhanced by an elevated level in the drinking water and Amyloid precursor protein (APP) in a transgenic (Tg) model of Alzheimer's disease. Mice were given water containing 10ppm $FeNO_3$ and lab tap water prenatally and from birth. After reaching one year of age animals were tested in a Morris water maze (MWM), a behavioral test of spatial learning and memory and assessed for behavioral deficits associated with Alzheimer's pathology and enhanced supplementation of iron. After completion of behavioral testing, mice were sacrificed and brains were harvested for histological examination to explore plaque formation. This was done using a Congo red stain and Image J software, which quantifies plaque load and plaque type using electron microscopy and a polarizing lens. Research showed that supplementation

of Fe in the drinking water had an impact on both plaque area (Tg mice only) and behavior in both groups of Fe treated mice. For MWM probe trial percent in quadrant Fe Tg mice spent more time in the correct quadrant than lab Tg, $p < .05$. Lab Wt mice spent more time in the correct quadrant than lab Tg, $p < .01$. For the moving platform, on day 1 Wt mice had faster escape latencies than Tg mice, $p < .01$, and on day 2 Wt mice also had faster escape latencies than Tg, $p < .01$. In addition, Fe mice had faster escape latencies than lab water treated mice, $p < .05$. Thus, overall these results indicate that Fe treated groups and Wt lab mice performed better in the MWM than the lab Tg group. Histological analysis revealed that lab Tg mice had more plaques than Fe Tg mice, $p < .01$. There was also a main effect of plaque type with more normal plaques than birefringent plaques, $p < .01$. This suggests that reduced plaque burden leads to a reduced impairment in the MWM. Recent information has pointed to a recessive blindness gene in the Tg2576 mouse model of AD. Swim patterns have been examined, and it is thought that some Wt mice with slow latencies may have been blind. As a result, current results are being further examined, and tails will be genotyped if possible. This is being done to determine if the data presented in this thesis are as accurate as possible. Preliminary analyses are reported here, but results may change once it is determined which if any animals were blind.

1. Introduction

Alzheimer's disease (AD) is a progressive degenerative disease of the central nervous system and the most common form of dementia in the elderly over the age of 65 (Alzheimer's Association, 2007). AD gradually destroys a person's memory, the ability to learn and reason, make judgments, communicate, and carry out daily activities. As AD progresses most individuals will experience changes in personality and behavior, such as anxiety, paranoia, agitation, and sometimes even delusions or hallucinations. Currently an estimated 5 million Americans have AD, a number that has doubled since 1980. It is estimated that 7.7 million will be afflicted by 2030 and, with no cure, 16 million by 2050 (NIA, 2007).

The greatest risk for AD is increasing age, with 1 in 10 over the age of 65 being affected and more than half of those individuals over the age of 85. In addition, there are also genetic or familial forms of the disease that can strike individuals as early as their 30's and 40's. Currently 200,000-500,000 Americans under the age of 65 now suffer from early onset Alzheimer's or some other form of dementia. In addition, with the enormous population of 78 million baby boomers, it is estimated that every 72 seconds in America someone develops Alzheimer's. It is projected that by mid century it will drop to 33 seconds (Alzheimer's Association, 2007).

Memory and Alzheimer's

The main characteristic of AD is loss of memory. Memory is a very important behavioral measure and very broad in scope, so the present experiment focused on spatial memory and its deficits associated with the progression of AD. The following section briefly overviews memory systems and the associated parts of the brain.

There are two types of memory: explicit or declarative and implicit or non-declarative (Squire & Zola, 1996). Declarative memory can be further broken down into two parts, semantic and episodic. Semantic memories are memories of facts, such as information that has been learned; the entorhinal cortex and the temporal lobe regulate this type of memory. Episodic memory is the memory for events, or autobiographical information, and is regulated by the hippocampus. The two major forms of non-declarative memory are procedural (habit learning), which is regulated by the basal ganglia, and priming a form of implicit memory, that is regulated by the primary visual cortex. It also includes spatial learning, regulated by the hippocampus, and fear learning, regulated by the amygdala (Squire & Kandel, 1999).

Memory deficits associated with AD are caused by a disruption in memory systems, seen initially in declarative forms of memory (Carlesimo & Oscar-Merman, 1992). This disruption is caused by the formation of plaques and plaque-associated degradation in the temporal lobes, including the entorhinal cortex and hippocampus. Subsequently, plaques are formed in the fusiform gyrus, which is responsible for facial recognition and complex visual stimuli. Later the amygdala, which regulates basic emotions such as fear, anger, and joy, is affected. Then AD affects the frontal lobes that

help carry out purposeful behaviors, complex reasoning, and working memory. Finally the areas associated with procedural learning, such as the basal ganglia, are affected (Braak & Braak, 1991). These plaques, in conjunction with environmental factors such as lifestyle (exercise, education, diet, and stress), contribute to the severity of dementia (Snowdon, 2002).

Plaques and Amyloid

Plaques are a biological indicator of AD, and they are formed by an aggregation of amyloid, which is made from the peptide amyloid precursor protein (APP) (Glenner & Wong, 1984). Although, everyone gets some plaque formation as they age, and it seems to be a normal part of senescence, it is much worse for individuals with AD. Research has indicated that amyloid comes in many forms, e.g. Aβ1-40 and Aβ1-42, with numbers indicating the number of amino acids in the peptide. The protein begins in a soluble form (Aβ1-40), which is initially absorbed or cleared away, but over time aggregates into insoluble forms of the protein, which become diffuse and dense-core plaques. Aβ1-42 aggregates into extracellular plaques more readily. As the disease progresses, the number and size of these plaques increase and the number of dense core plaques increase, leading to the decline in cognitive and emotional functioning associated with the disease (Braak & Braak, 1997).

Elevated levels of trace metals such as zinc, iron, and copper have previously been reported in β-amyloid (Aβ) plaques and adjacent tissue in AD (Lovell, Robertson, Teesdale, Campbell, & Markesbery, 1998). Currently, research has been giving increased attention to the role of these metals in amyloid aggregation and how this relates

to the progression of AD. This is important because copper, zinc, and iron are concentrated in and around amyloid plaques in the AD brain, and high levels of zinc and iron have been reported in the amyloid plaques of both humans and the Tg2576 mouse model for AD (Maynard, Bush, Masters, Cappai, & Li, 2005). However, the role of the individual metals is not clear, and needs further examination. Thus, the purpose of the current study was to examine the role iron plays in the progression of AD and its effects on formation and aggregation of amyloid.

The Role of Iron

Iron is crucial for the brain's normal development and also for metabolic functions, energy metabolism, and synthesis of neurotransmitters (Beard, Connor, & Jones, 1993). During brain development, iron is present in white matter where high levels are found in oligodendrocytes that are required for the production of myelin (Beard et al., 1993). Iron in the brain is also contained in enzymes, structural proteins, transport proteins such as transferrin, and in storage proteins such as ferritin (Burdo, Antonetti, Wolpert, & Connor, 2003). Levels of iron in the brain increase with age, and different cerebral regions accumulate iron at both different rates and concentrations (Hill, 1988). In adult human brains the highest concentrations of iron are found in the basal ganglia (specifically the globus pallidus), the red nucleus, and the substantia nigra (Koeppen, 2003).

Iron is the most abundant transition metal in the brain, and in biology in general, and it is considered the most potent potential toxin, because it has the ability to do the most damage via oxidation (Thompson, Shoham, & Connor, 2001). However, the brain

needs a constant supply of iron for transport, storage, and regulation. If, for some reason, the brain fails to meet the demands for iron in a proper working manner, persistent decline in neurological and cognitive function can occur.

The blood brain barrier (BBB) and blood cerebrospinal fluid (CSF) barrier help to control the iron uptake into the brain. This is done by the regulation of the genetic expression of transport protein receptors. Both the BBB and CSF barrier maintain this control by regulating transferrin receptor expression in endothelial and choroids plexus cells (Burdo et al., 2003). In the brain, iron does not produce toxicity, even though it is found in high levels. This is probably due to the efficient homeostatic process that is maintained with normal brain function. The system's homeostatic status is quite sensitive and if there is a disruption of the status, then iron-induced oxidative damage can occur (Bush, 2003).

Defective iron homeostasis, resulting in increased iron levels in the brain, is commonly seen in AD (Poduslo, Wengenack, & Curran, 2002; Poduslo, Wengenack, & Curran, 2001) and other neurodegenerative disorders (Gelman, 1995), including Parkinson's and thalassemia (Thompson, Shoham, & Connor, 2001). However, AD patients demonstrate more iron in the neuropil than patients who do not have AD (Lovell et al., 1998). Aβ protein has been discovered to have metal-ion binding sites and the direct interaction that occurs between Aβ and iron is a factor considered to be responsible for the aggregation and accumulation of Aβ (Bishop et al., 2002). However, the exact role of iron in the pathogenesis of AD is not yet completely clear. It is quite possible that a breakdown of metal regulation could be an inevitable part of the aging process.

In the AD brain, iron appears to be particularly concentrated in amyloid plaques and is suspected of catalyzing the formation of free radicals (Markesbery & Carney, 1999). This strongly suggests that high levels of iron are directly related to, if not causal, in the formation of amyloid plaques. Aβ can produce hydrogen peroxide (H_2O_2), a source of free radicals, which can lead to tissue damage in a copper/iron dependent manner (Huang et al., 2000). This reaction occurs when Aβ reduces Cu^{2+} to Cu^{1+} or Fe^{3+} to Fe^{2+}, and acts as a catalyst for the oxygen dependent production of H_2O_2, or reactive oxygen species (ROS), which may result in cellular toxicity and initiate cell death.

Over the course of the normal aging process the number of ROS in the brain increases, as do levels of trace metals (Christen, 2000). Because the central nervous system (CNS) is susceptible to damage by ROS this should be considered very important. Trace metals (copper, iron, and zinc) are often implicated as the mediators of oxidative stress and ROS in the production of neurodegenerative diseases (Sayre, Perry, & Smith, 1999). Current research is beginning to show that metallochemical reactions resulting in the formation of ROS are a common factor underlying AD (Bush, 2000).

Evidence suggests that when Aβ binds with iron it (Aβ) may act as a chelator to diminish oxidative damage caused by high concentrations of iron (Bush, 2003; Todorich & Connor, 2004). This is best described in a model proposed by Bush and Tanzi (2002) and is shown below in Figure 1.

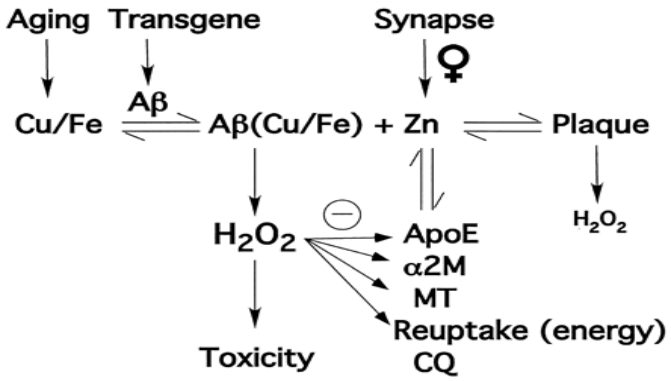


Figure 1: Ashley Bush Model

The model proposes that plaques exist due to a relationship between free Zinc (Zn^{2+}) and soluble Aβ, which may be bound to copper (Cu^{2+}) or Iron (Fe^{3+}). According to the model, Zn^{2+} is supplied by synaptic activity from the glutamatergic fibers of the corticofugal system. The Zn^{2+} is re-assimilated into the neuron by an energy-dependent reuptake system. Zn^{2+} may also be sequestered by apolipoprotein E (ApoE), α-2-macroglobulin (α2M), metallothioneins (MT), or by chelating drugs such as clioquinol (CQ). The oxidizing effects of excess H_2O_2, generated by Aβ, inhibit the ability of several biochemical factors to lower the concentration of free Zn^{2+}. Zn^{2+} rich plaques produce less H_2O_2 than Zn^{2+}-free Aβ. Additionally, Cu and Fe levels rise in senescence. Females undergo increased synaptic release of Zn^{2+} with age (Bush & Tanzi, 2002). This suggests that accumulation of amyloid and the change in rates of iron metabolism appear to contribute to oxidative stress. It also implicates the interaction of amyloid and iron as potential mechanisms for changes that relate to the neurodegeneration associated with the progression of AD.

A study conducted at Harvard University Medical Center (Huang et al., 2004) supports the Bush model and demonstrates that Aβ is a high-affinity metalloprotein that aggregates in the presence of biometals including zinc, copper, and iron. In the study, a chelator (DTPA) was used in rats in vivo to remove metals from the brain. The introduction of DTPA stopped the Aβ seeding (or growth) process and consequently plaque formation. The findings of the study suggest that these trace metals (particularly iron), are the actual initiators of Aβ-42 mediated seeding; i.e. the start of the protein formation reaction and the oligomerization process, which is the maturation of the protein. The results directly link exogenous metal ions, specifically iron, to the Aβ seeding and oligomerization process. This suggests there is an essential role for these metals in initiating Aβ formation and aggregation in the brain.

In AD it appears that iron has a direct effect on Aβ deposition, specifically in transcription and translation of amyloid precursor protein (APP) (Christen, 2000), and in ROS formation (Varadarajan et al., 2000). APP contains an iron response element (IRE), suggesting that the synthesis of the amyloid protein is directly influenced by cellular iron levels (Rogers et al., 1999). IREs are the RNA stem loops that control cellular iron homeostasis (Zubenko, Farr, Stiffer, Huges, & Kaplan, 1992). For APP, IREs control the rate at which APP is made. This is done by the regulation of ferritin translation and transferrin receptor mRNA stability (Eisenstein & Theil, 2000).

Disruption in the stability and production of proteins has a direct effect on the sensitive homeostatic process in which iron must be maintained in order for normal function to occur. Rogers et al. (2002) demonstrated this by mapping a novel iron-

responsive element (IRE-Type II) within the 5'-untranslated region of APP transcript. The study showed that translation was selectively down-regulated in response to intracellular iron chelation. Thus, iron regulation of APP mRNA points to a role for iron in the metabolism of APP, and indicates that increased iron leads to an increase in APP. This could lead to an increase in plaques.

Research Implications

Iron levels may not ethically be administered to humans for research purposes but such studies can be done in rodents. For example, research using transgenic (Tg) mice supports this notion of iron playing such a critical role in AD. The PS/APP mouse is a Tg mouse model of AD over expressing both human APP and presenilin 1 (PS1) (McGowan et al., 1999). As a result the mice show early and extensive amyloid deposition. Falangola et al. (2005) confirmed the presence of iron co-localized with Aβ plaques, in PS/APP mice brains, using Perls' stain (Prussian blue) for Fe^{3+}. Additionally, histological sections fixed with formalin showed the presence of iron in the majority of Aβ plaques. This observation of iron accumulation in Aβ plaques in PS/APP mice (Falangola, et al., 2005) is consistent with previous studies in humans, which also demonstrated plaque and iron co-localization (Connor, Menzies, St. Martin, & Mufson, 1992; Connor, 1997). The histological detection and analysis of iron in Aβ plaques is important because of its role in the pathogenesis (development and progression) of the disease.

In conjunction with those findings, Thompson et al. (2005) compared the dietary enhancement in the drinking water of zinc or iron in Tg2576 mice. The Tg2576 mice

carry the human APP gene and show plaque formation and cognitive impairments in spatial and working memory at 9 months of age (Hsiao et al., 1996). In the study, mice were given water containing 10ppm $ZnCO_3$, 5ppm $FeNO_3$, or lab tap water. At 12 months of age the animals were tested in a MWM, then brains were harvested and flash frozen and stored at -80° C to await in situ hybridization. This is a histological method in which radioactive probes are used to label mRNA of certain proteins, in this case APP. The results of the study (Thompson et al., 2005) showed that even low levels of trace metals in the drinking water could significantly affect APP mRNA levels within the hippocampal region. This is important because the hippocampus (HC) is essential for the formation of new memories and for the acquisition of spatial tasks. Amyloid deposition in this area of the brain can disrupt normal function and directly affect learning, memory, and emotion.

In addition, animals demonstrated a pattern of circling behavior in a Morris water maze (MWM) that suggested there was a disruption in their normal motor function. This suggested possible iron deposition in the basal ganglia (BG), due to observed impairments in motor function, and further implicated evidence for iron's contribution to diseases of the BG such as Parkinson's, which is also amyloid related (Youdim, Ben-Shachar, & Riederer, 1990). Previously, Linkous et al. (2004) demonstrated histological changes in plaque formation of Tg mice using dietary enhancement of both zinc and iron in the drinking water. Tg mice in the Fe treated group had more plaques in a region of the HC than zinc Tg, or lab Tg mice. This strengthens the connections of iron and further implicates its role in the regulation of APP and amyloid aggregation.

A related study conducted by Frackowiak et al. (2003) found that brain vascular smooth muscle cells from Tg2576 mice over-expressed the APP transgene in culture, secreted amyloid-B peptide (Aβ), accumulated Aβ intracellularly, and were enhanced by iron (D'Andrea, Nagele, Wang, Peterson, & Lee, 2001). This intracellular Aβ, partially aggregated and oligomerized, was proposed to be cytotoxic and to initialize the seeding and aggregation of extracellular Aβ (Gouras et al., 2000). The mechanisms leading to precipitation of the soluble Aβ into intracellular and extracellular amyloid deposits are not yet clear. However, it is known that an increased production of Aβ-42 is very important for amyloid deposition (Mazur-Kolecka, Kowal, Sukontasup, & Frackowiak, 2003).

Formation of Aβ fibrils (which collectively become plaques) appears to be influenced by multiple factors that affect the solubility and clearance of Aβ, and locally promote its aggregation (McLaurin, Yang, & Frasier, 2000). Such factors include apolipoprotein E (Mazur-Kolecka, Frackowiak, & Wisniewski, 1995), and oxidative stress (Mazur-Kolecka, Frackowiak, Kowal, Krzeslowska, & Dickson, 2002). Aggregation and fibrillization of Aβ in vitro is enhanced by iron (Atwood et al., 1998) and may be induced by free radicals generated by H_2O_2. Therefore, increased concentrations of iron in amyloid deposits in the AD brain may be interpreted as due to iron-enhanced aggregation of Aβ (Bush et al., 1994). Therefore high levels of iron in the brain should be considered a major risk factor in the acquisition of AD.

Lysosomes and Neuronal Damage

Lysosomes are organelles that contain enzymes that are used to digest macromolecules. The lysosomes are used for the digestion of macromolecules from phagocytosis (ingestion of cells), from the cells own recycling process, and for autophagic cell death. This is a form of programmed self-destruction, or autolysis of the cell; which means that the cell is digesting itself. Other functions include digesting foreign bacteria that invade a cell, and helping to repair damage to the plasma membrane by serving as a membrane patch, thus sealing the wound.

The enhanced lysosomal deposition of Aβ, which is seen in cells, treated with iron (Cole, Huyn, & Saitoh, 1989) points to a factor that may trigger the process of amyloid deposition in blood vessels. This is an increased availability of iron in the local vascular environment, caused either by altered metabolism of iron or by a release of iron from micro-hemorrhages. Whether or not iron could also directly affect aggregation of Aβ in lysosomes has yet to be established. The enhanced intralysosomal accumulation of Aβ, in cultures treated with ferrous ions, indicates that an increased availability of iron ions in the walls of blood vessels in the brain may be the trigger that promotes the formation of vascular amyloid (Cole et al., 1989).

Amyloidogenic effect of ferrous ions may be related to oxidative stress caused by the redox active ions, because Aβ deposition has been found to be correlated with cellular levels of oxidation-modified proteins (Mazur-Kolecka et al., 2002). The findings of Frackowiak et al. (2004) showed that in contrast to iron, copper, zinc and aluminum have little effect on intracellular accumulation of Aβ. This accumulation is a marker of cell

damage caused by oxidative stress, and indicates that iron targets the proteins crucial for Aβ accumulation extracellularly. The effect of iron on intracellular deposition of Aβ could also be associated with a direct stimulation of Aβ aggregation in lysosomes, as it was shown to act on Aβ in cell free systems (Mantyhet et al., 1993). Frackowiak's (2004) findings suggest that intra-lysosomal accumulation of Aβ and APP may be an early step in deposition of vascular Aβ in Tg2576 mice.

Accumulation of both Aβ and APP in the lysosomes suggests that Aβ present in vascular smooth muscle cells from Tg2576 mice is generated by lysosomal degradation of APP. Aβ has already shown to be generated by lysosomal processing of APP in neurons of humans and rodents (Leblanc & Goodyear, 1999). Accumulation of Aβ in lysosomes may have profound pathological consequences. Aβ1-42, soluble when aggregated may disrupt the integrity of the lysosomal membranes in neurons (Ditaranto, Tekirian, & Yang, 2001), resulting in leakage of lysosomal enzymes, damage of cell cytoskeleton, and eventually cause cell death (Opazo et al., 2002). In contrast to amyloid plaques, that contain mainly Aβ1-42, the vascular amyloid is composed mainly of Aβ1-40 (Miller et al., 1993). However, vascular smooth muscle cells in the vicinity of amyloid deposits also degenerate, as shown in vivo in humans (Vinters et al., 1994), and in APP-Swe mice (Christie, Yamada, Moskowitz, & Hyman, 2001).

These findings coincide with recent identification of APP as one of the resident lysosomal membrane proteins (Pasternak et al., 2003). Enhancement of cellular accumulation of Aβ by treatment with iron ions points to redox active iron in the brain in vivo as a risk factor for amyloidogenesis. Induction of Aβ deposition by iron was also

recently shown using histological examination in non- transgenic vascular smooth muscle cells, of humans, and canines (Frackowiak et. al, 2004). It remains to be seen whether Aβ deposition can be triggered by iron in other types of cells.

Inflammation

In a recent study, Ong and Farooqui (2005) examined the roles and interactions of iron, histologically, and how it relates to neuroinflammation seen in AD, using a mouse model. This study examined the importance of neuroinflammatory changes in the glial scar, after neuronal injury, in promoting iron accumulation and iron-dependent oxidative damage. Results demonstrated that elevation of iron in AD, not only affects APP processing and mitochondrial function, but also induces the aggregation of Aβ and abnormalities in signal transduction processes associated with oxidative damage. This evidence suggests that although alterations in iron homeostasis may not be the only triggering event that starts the pathological cascade of Alzheimer's disease, it is an important factor involved in neuroinflammation and progression of this disease.

Hemochromatosis

In addition to animal studies, it is also important to look at literature focusing on how this information about iron can be generalized to humans. This particular line of research focuses on mutations in a gene known to be important in the development of hemochromatosis, or “iron overload” disease, which has been shown to be a contributing factor for Alzheimer's disease in some patients (Connor et al., 2001). This is a definite area of concern, most importantly because iron overload can be controlled. This means

there could be the potential for immediate therapeutic actions to delay AD onset and/or progression in affected people.

Hemochromatosis is a condition resulting from excessive uptake of dietary iron, which is subsequently deposited in the liver, heart, pancreas and other organs. It is the most common genetic disease so far identified, with around 1 in 200 people severely affected. Most people with hemochromatosis have mutations in the HFE gene, discovered in 1996 within the major histocompatibility (MHC)/HLA locus on the short arm of chromosome 6 (Connor et al., 2001), and named by the WHO Human Gene Nomenclature Committee.

The HFE protein is proposed to interact with the transferrin (Tf) receptor to regulate cellular iron uptake. Mutations leading to abnormal HFE protein can dysregulate uptake, causing cellular overloading. Iron uptake into the brain by transcytosis across the BBB and at other sites is likewise carried out by the Tf receptor. Evidence has now been reported (Connor et al., 2001) that the HFE protein is found on brain capillaries, choroid plexus, and ependymal cells, along with Tf receptors. As a result, iron uptake in the brain could be influenced by HFE mutations. Furthermore, it has been reported that in AD, the HFE protein appears to be induced on neurons, on the cells associated with neuritic plaques, and on astrocytes associated with blood vessels.

Most patients with severe hemochromatosis are homozygous for the major C282Y mutation of the HFE gene. This mutation has an allelic frequency of 1-15% in Caucasian populations, being frequent in populations of Celtic ancestry but less common in Mediterranean countries. The second common mutation is H63D (allelic frequency 10-

20%), which usually has less severe effects on iron status than C282Y. Many homozygotes and some heterozygotes, particularly C282Y/H63D compound heterozygotes, will develop clinical hemochromatosis with aging. Overall estimates suggest 20-40% of people with European ancestry carry at least one mutant HFE allele. Because these mutations are so common, a significant percentage of AD patients can be expected to carry one or more HFE mutations.

Although hemochromatosis has not been traditionally associated with brain iron loading, this is now being examined. While in the past, many hemochromatosis sufferers did not survive past their 40s or 50s, most patients now have normal life spans due to improved treatment. These people may develop brain conditions, such as AD, that previously would not have been identified. Furthermore, iron loading might eventually have consequences, due to accumulation as the lifespan increases.

It has been recognized for many years that iron accumulates in the AD-affected hippocampus and in other severely affected brain regions, in association with neuritic plaques and neurofibrillary tangles. While so far the relevance of this iron accumulation to the pathogenesis of AD has been uncertain, there are various feasible mechanisms by which iron excess could contribute to AD. One simple hypothesis is that iron overload superimposes on other AD processes to accelerate brain damage and exacerbate symptoms, possibly by amplifying oxidative damage to neurons.

In addition, three studies have now found associations between HFE gene mutations and dementia. In a North American study, men lacking both the apoE4 allele and the two major HFE mutations were less likely to have familial Alzheimer's disease

(Moalem et al., 2000). Many of the symptoms of iron overload manifest more severely in men than in women. This may be associated with protection of iron overload in women by menstruation and estrogen. Carrying of one or more allelic copies of the major C282Y mutation was significantly associated with early onset AD (50-65 yrs). In addition, Sampietro et al., 2001, have reported that in an Italian sample, where the C282Y mutation is very rare, onset of AD occurred about 5 years earlier in subjects carrying one or more copies of the H63D mutation, independent of gender. In patients under 70 years at AD onset, the frequency of the H63D mutation was five times higher than in those over 80 years at onset. These studies suggest that not just homozygosity but also heterozygosity for the main HFE mutations may influence AD pathogenesis. If this is true, as many as 25% or more of Americans and others of European descent could be affected.

This information shows that the iron accumulation occurs in the brain and is associated with amyloid production and aggregation (Bush et al., 2004; Huang et al., 2004). The homeostasis of iron in the brain is a sensitive matter and minor fluctuations can produce major changes such as ROS, alterations in transcription and translation of APP, and the initiation of Aβ aggregation (Maynard et al, 2002; Bush et al., 2002, Rogers et al., 2002). These changes are arguably directly related to AD, and changes in iron levels appear to play a major role in neurodegenerative damage and progression. Whether iron could be a causative agent in AD through direct effects on Aβ and APP processing, defects in iron export from mitochondria, disrupted intracellular iron trafficking, or by promoting cell death through oxidative stress is still not clear. However, it does appear that these possibilities are not mutually exclusive. What is clear

is that iron can affect the disease process of AD and that more research is needed concerning iron homeostasis and its link to AD progression by assessing MWM performance and plaque area.

Rationale for the Present Study

The cellular work with iron in the literature is quite abundant, but there is no real consensus on how iron is directly related to AD pathogenesis, except that it does play a critical role. In particular one aspect of research that is clearly lacking is behavioral data. More research is needed that investigates links between both the physiological and the behavioral aspects of iron's role in AD. Tests such as the Morris water maze (MWM), which measures spatial memory, could help to fill in the gaps and answer questions about how these changes seen in the brain affect learning and memory processes. The MWM, developed by Richard Morris (1984), is a circular pool with a platform-submerged 1cm under the water. Animals are placed at different locations in the pool and the task is for the animal to find the submerged platform after training (subsequently learning its location) across a succession of trials.

The task was designed to test spatial reference and working memories, which are hippocampal dependent. Thus, if the animals have no deficits associated with spatial memory systems they learn the task quite successfully in a short time. However, animals with compromised spatial memory systems will perform more poorly and will take longer to learn the task or will not successfully master the test at all (ibid). A behavioral test such as the MWM is an ideal tool and is relevant to the assessment of deficits associated with AD pathologies as they extend to hippocampal and basal ganglia damage. Therefore,

the MWM was chosen to determine what deficits, if any, will occur as a result of dietary enhancement of iron in the drinking water in an effort to develop a better understanding of iron's role in AD progression, through the assessment of MWM performance and plaque measurements.

Hypotheses/Specific Aims

This study sought to further explore the relationship between iron and APP through prenatal dietary enhancement in the drinking water (Flinn et al., 2005), in an effort to determine iron's role in the progression of AD. As stated before, preliminary data (Thompson et al., 2005) suggests iron does perform a critical role in regulation of APP gene expression and has an effect on animal performance in behavioral tests (MWM). The present experiment, using a mouse model of AD, hoped to demonstrate iron's regulatory role in APP plaque formation, and subsequent behavioral deficits as a result. It was hypothesized that iron would increase plaque formation in Tg animals raised on $FeNO_3$. It was also hypothesized that, in general, animals raised on $FeNO_3$ would perform more poorly in behavioral tasks (MWM) than animals raised on tap water.

2. Methods

The experiment used male and female Tg2576 (n =16) and Wt2576 (n=18) animals were prenatally exposed to 10pppm $FeCl_3$ and then raised on water containing 10ppm $FeNO_3$ or lab tap water (see below). Starting at 12 months of age, animals underwent behavioral testing using a Morris Water Maze (MWM) to examine any deficits associated with iron consumption. After behavioral testing was complete, animals were sacrificed and brains were harvested for histological examination. Brains were fresh frozen in dry ice and then stored in an -80°C freezer. Histology was done using Congo red to determine levels of AD like plaques in the brain. Statistical analyses were performed using repeated measures ANOVA. The purpose of the experiment was to examine whether raising mice on iron-enriched water would increase the number of plaques of Tg2576 animals as opposed to those raised on tap water. In addition statistical analyses of behavioral tests examined whether animals raised on iron-enhanced water demonstrate greater memory impairments in Tg groups, but not in Wt groups.

Subjects

Male and female Tg2576 (n = 16; lab (n = 9); (male = 4, female = 5) and iron (n = 7); (male = 2, female = 5)) and Wt2576 (n= 18); lab (n = 9); (male = 4, female = 5) and iron (n= 9); (male = 5, female = 4)) bred at Mason, prenatally on water containing 10 ppm $FeCl_3$ or lab tap water. $FeCl_3$, was used to prevent blue baby syndrome. After birth

mice were raised on water containing 10ppm $FeNO_3$, or tap water. Tg2576 and Wt2576 mice were bred and raised at George Mason University, on 10ppm $FeNO_3$ water (n=16) or tap water (n=18). Only brown-eyed animals were used, due to a priori knowledge of red-eyed animals having poor vision from the first group run at GMU (cohort 1). Food and water were administered ad libitum from birth. Lab treated groups produced more pups than Fe treated groups. Therefore, no litters had to be culled to provide comparable sample sizes. Animals were genotyped later and extra lab Wt mice were used in pilot work for future groups. There were not as many Tg animals as expected, as a result, Fe groups were smaller than lab groups resulting in a small N. Animals were housed three to a cage and kept under a 12-hour light/dark cycle. Tg males were singly if violent behavior (biting) towards cage mates was observed.

Materials:

Morris Water Maze

The mice were tested at 12 months of age in the Morris Water Maze (MWM), (Morris, 1984) to test spatial, reference, and working memory. The MWM may be used to measure both reference (submerged Atlantis platform) and working memory (moving platform), as discussed below. The MWM used was a 4' in diameter pool, with a transparent plexi-glass platform submerged 1cm beneath the water surface. White curtains surrounded the maze with black cues posted on the curtains on each side of the pool.

In order to observe reference memory, which is valid on all trials, an Atlantis platform was used. This platform is left in the same quadrant for every trial, but can be

submerged completely under the water. When the platform is not moved it is called "stationary" and when the platform is submerged this is referred to as a "probe" trial. The Atlantis paradigm lasted for 8 days, with 3 trials per day, and on every 6^{th} trial the platform was submerged and then was returned to its original position at the end of the trial. Each trial lasted for duration of 60 secs in which time the animal should find the platform. If the animal was successful in finding the platform it was given 10 seconds on the platform at the end of the trial. However, if the animal was not successful in completing the task they were gently guided to the platform and left there for a period of 15 seconds. After each trial the animals were returned to their cage for a rest period of 45 seconds.

Following the 13 days of Atlantis trials the animals were given 2 days of visible cue trials. This was to ensure that animals did not have sensory motor deficits. During these visible days the platform was raised out of the water and placed in different quadrants with a visible cue hanging directly over the platform. This was done with 2 trials per quadrant, 45 secs apart, for all 4 quadrants, with the Atlantis platform location last.

In order to test working memory, a moving platform paradigm was used. Working memory measures how well the animal can take the information it has learned from a previous trial and learn that the platform is now in a new location. Working memory trials were run for a period of 4 days and the platform was positioned in a new quadrant for each of the four days. Each day consisted of 4 trials of 60 secs/day with intervals of 2 minutes between trials A and B, and 20 secs between trials B and C, and C

and D. This was done to determine how well the animals remembered where the platform had been moved to each day. The behavior was recorded using HVS Watermaze 2020 Plus Module software and computerized tracking system. The MWM analysis will be done using repeated measures analysis of variance (RMANOVA); using various different designs based on water, genotype, and task specific measures (as explained in further detail in the results section).

Histological Analysis

After completion of all behavioral testing, mice were decapitated for brain extraction. Once brains were harvested they were flash-frozen in dry ice and stored at -80°C. Coronal sections 20µm in thickness were collected through the hippocampus, surrounding cortex, and the basal ganglia. Once slicing was complete sections were stained using Congo red to determine plaque levels as follows:

1. Mayer's Hematoxylin for 2-3 minutes.
2. Wash in tap water until blue.
3. Working (in solution) sodium chloride solution, allow slides to sit in solution for 2-5 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.

Staining yielded the following results, amyloid stained red to pink, and nuclei stained blue. Sections were then observed using an Olympus BX51 fluorescent/polarizing microscope and imaging system. Images were analyzed using the NIH public domain

image processing program ImageJ which can display, edit, analyze, process, and save microscope images, and can be used to calculate the area of user defined regions. This area is calculated in pixel values based on color and is an arbitrary measurement.

Congo red regions were quantified in terms of plaque burden (mean area of plaques). Also plaque types were examined looking at normal plaques and other plaques that under polarized light caused birefringence (plaques turn green). This birefringent plaque demonstrates the presence of beta-pleated sheets, which are seen in advanced stages of AD. The percent area loaded with plaques was measured from the hippocampus and cerebral cortex at 100μm intervals. The regions of interest (ROI) specifically examined were as follows (refer to appendix for images of individual regions examined): ROI 1 (ventral hippocampus), ROI 2 (dorsal hippocampus), ROI 3 (anterior hippocampus), ROI 4 (basal ganglia 1), and ROI 5 (basal ganglia 2). Differences between groups were assessed by a 2 (water) X 5 (region) RMANOVA; a 2(water) X 3(region) RMANOVA; and a 2 (water) X 2 (type) X 5 (region) RMANOVA using a significance level of $\alpha = .05$. The, Congo red, measurements were taken by two separate raters that demonstrated high inter-rater reliability.

3. Results

For MWM Atlantis Platform paradigm 3 trials a day were conducted for 8 days. However, because the first day is for training only and the second day contains the first probe trial, which are analyzed separately, these days were not included in the final analyses for A and B trials (refer to appendix for all Atlantis platform latency data). In addition, since behavior is expected to plateau in the final two days (7 & 8), these days were also not included. Therefore, days 3-6 were analyzed using a 2 (Water: iron, lab) X 2 (Genotype: Tg2576, Wt2576) X 2 (Trial: A, B) X 4 (Days) RMANOVAs. There were no significant differences in escape latency (length of time needed to find the platform), Gallagher proximity measure (the average distance from the platform), or thigmotaxicity (a measure of anxiety, defined as the percent of time spent swimming in the outermost 10% of the pool closest to the edge) for either the A or B trials.

For Probe trials (Trial C), 2 (water) X 2 (genotype) X 4 (days) RMANOVAs were conducted on percentage of time spent in the platform quadrant, Gallagher measures, and platform crossings. For percentage of time in quadrant there was a significant interaction between Water and Genotype ($F(1, 30) = 4.886$, $p = .035$) (refer to Table 1 and Figure 2). A simple effects analysis revealed that Fe Tg animals spent more time in the correct quadrant than lab Tg animals ($F(1, 30) = 6.25$, $p < .05$) and lab Wt spent more time in the correct quadrant than lab Tg ($F(1, 30) = 9.33$, $p < .01$). There was also a trend for a main

effect of Genotype (see Figure 3) ($F(1, 30) = 4.886$, $p = .058$), with Wt performing better than Tg, as expected.

For the Gallagher measure, there was a significant main effect of Genotype ($F(1, 30) = 4.223$, $p = .049$). Unexpectedly, Tg animals swam significantly closer, on average, to the platform than Wt (refer to Table 2 and Figure 4) and there was a trend toward a significant interaction of water by genotype ($F(1, 30) = 3.557$, $p = .069$) with lab Tg animals swimming closer on average to the platform than lab wt (see Figure 5). There was no significant difference for the Fe groups. Platform crossings analysis yielded no significant results. A visible platform check, including a 2 (water) X 2 (genotype) X 2 (day) X 2 (trial) RMANOVA, and examination of swim patterns for visual deficits and behavioral anomalies showed no significant differences between groups (refer to appendix for visible platform data).

For the Moving platform paradigm, measuring working memory, escape latency data were analyzed using a 2 (water) X 2 (genotype) X 4 (trials) X 4 (days) RMANOVA (for moving platform escape latency descriptive statistics refer to Tables 3, 4, 5, & 6). There was no significant escape latency effect for trials. There was a significant interaction of day by genotype ($F(1, 30) = 3.247$, $p = .026$) (see Figure 6). Simple effects analyses revealed that on day 1 Wt animals had faster escape latencies than Tg ($F(1, 30) = 10.50$, $p < .01$), and on day 2 ($F(1, 30) = 7.88$, $p < .01$) Wt animals had faster escape latencies than Tg. There were no significant differences between Wt and Tg animals in escape latencies on days 3 and 4. In addition, there was a main effect of water type ($F(1, 30) = 6.953$, $p = .013$). Iron animals had faster escape latencies than lab (see Figure 7)

and there was a trend for an interaction of day by water by genotype. Thus, overall, these results indicate that Fe treated groups and the Wt lab mice performed better than the lab Tg group.

Histological analyses were performed on Tg brain tissues (refer to appendix for images of regions). A 2 (Water) X 5 (Region of interest: HC: 1, 2, & 3; BG: 4 & 5) RMANOVA yielded no significant difference in plaque burden (average area of plaques) across regions between water groups (refer to Table 7 for Means and SD's of Plaque Areas). However, when excluding region 1 and region 2 of the HC from the final analysis (as iron was expected to have less of an effect in these regions of the brain), a 2(water) X 3 (regions) RMANOVA comparing the most anterior part of the HC, with the regions of the BG found a significant main effect of water ($F(1, 14) = 9.633$, $p = .008$). Lab Tg mice had significantly more plaques than Fe Tg mice (see Figure 8). The same 2 (water) X 3 (regions) RMANOVA conducted on birefringent (beta pleated sheets) images yielded a trend for a main effect of water type, ($F(1, 14) = 3.977$, $p = .066$) with lab Tg having more plaques than Fe Tg (see Figure 9).

In addition, a separate analysis was conducted comparing plaque type 2(Water) X 2 (type: normal plaques and birefringent plaques) X 5 (regions of interest: HC = 1, 2, &3, and BG 4 & 5), and there was a significant main effect of location ($F(1, 14) = 2.792$, $p = .035$). Tukey post hoc tests did not reveal any significant differences. However, the RMANOVA revealed that there were more plaques overall in the HC than in the BG (for a complete breakdown of this effect see Figures 10, 11, 12, & 13). There was also a main effect of plaque type ($F(1, 14) = 89.831$, $p < .001$) with more non-birefringent plaque

areas than birefringent (beta pleated sheets) plaque areas (see Figure 14). This indicates that the Fe treated group had less plaque area development than the lab treated group, particularly in regions 3, 4, and 5.

Table 1: Means and standard deviations for probe trial percent in quadrant data Days 2, 4, 6, & 8.

	Water	Genotype	Mean	Std. Deviation	N
Day 2	lab	**WT**	23.80	15.56	9
		TG	13.61	15.93	9
	iron	**WT**	32.711	16.48	9
		TG	29.757	24.23	7
Day 4	lab	**WT**	30.056	18.33	9
		TG	18.089	19.53	9
	iron	**WT**	24.811	15.13	9
		TG	24.500	5.08	7
Day 6	lab	**WT**	38.844	16.07	9
		TG	11.589	11.17	9
	iron	**WT**	27.556	15.11	9
		TG	29.014	25.41	7
Day 8	lab	**WT**	26.60	21.11	9
		TG	16.58	21.43	9
	iron	**WT**	23.49	16.54	9
		TG	28.70	16.79	7

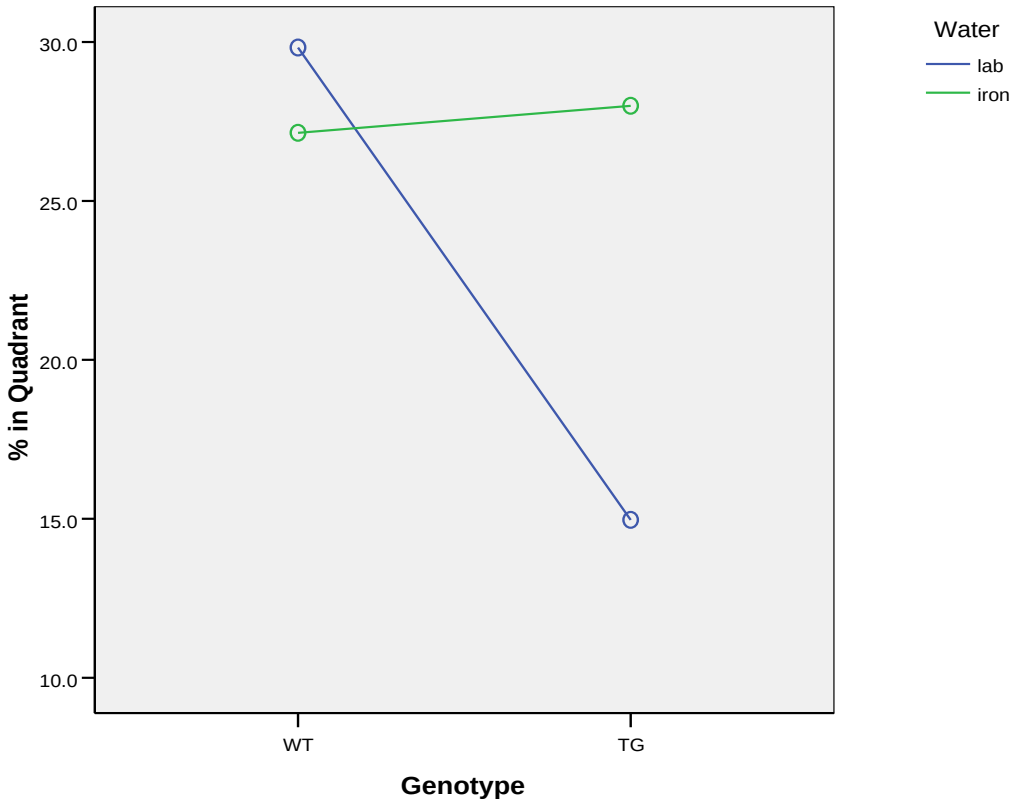


Figure 2: Probe trial percent in quadrant interaction of Genotype X Water

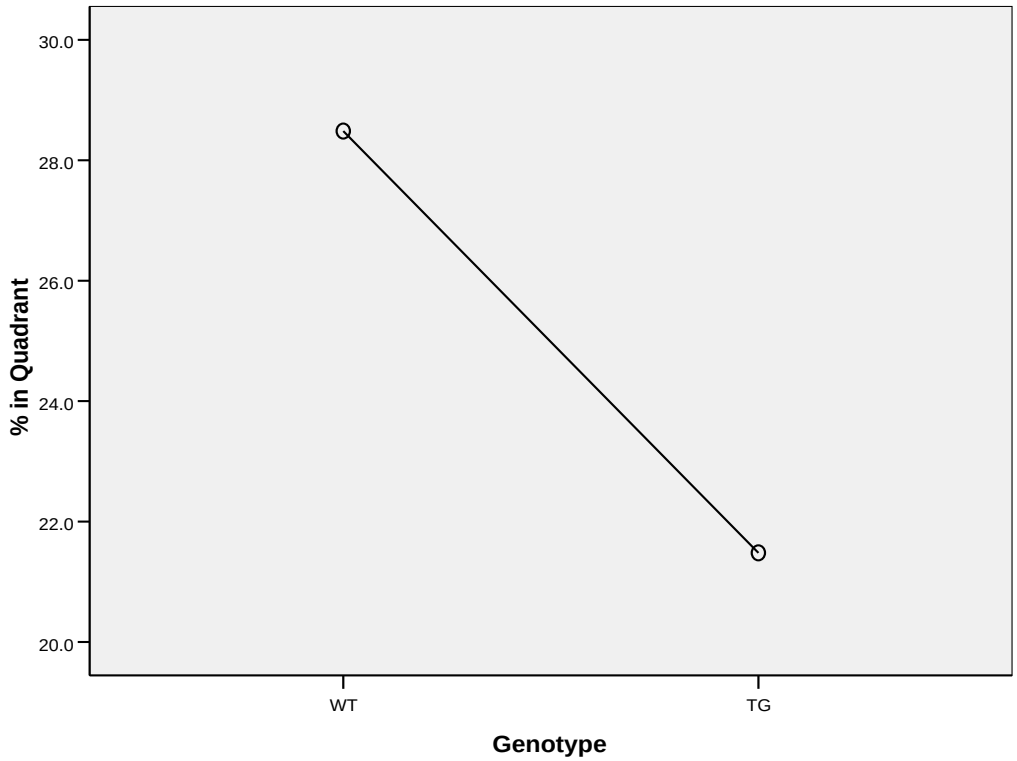


Figure 3: Probe trial percent in quadrant main effect of Genotype

Table 2: Means and standard deviations for probe trial Gallagher measures (distance from platform in cms) Days 2, 4, 6, & 8.

	Genotype	Water	Mean	Std. Deviation	N
Day 2	WT	lab	29.76	15.77	9
		iron	38.88	16.67	9
	Tg	lab	19.55	20.93	9
		iron	36.07	29.06	7
Day 4	WT	lab	33.81	18.89	9
		iron	29.52	16.32	9
	Tg	lab	20.52	21.60	9
		iron	28.71	7.08	7
Day 6	WT	lab	45.17	17.66	9
		iron	35.21	19.14	9
	Tg	lab	15.93	15.50	9
		iron	31.30	28.48	7
Day 8	WT	lab	32.52	25.83	9
		iron	29.68	22.64	9
	Tg	lab	18.97	23.49	9
		iron	34.37	20.53	7

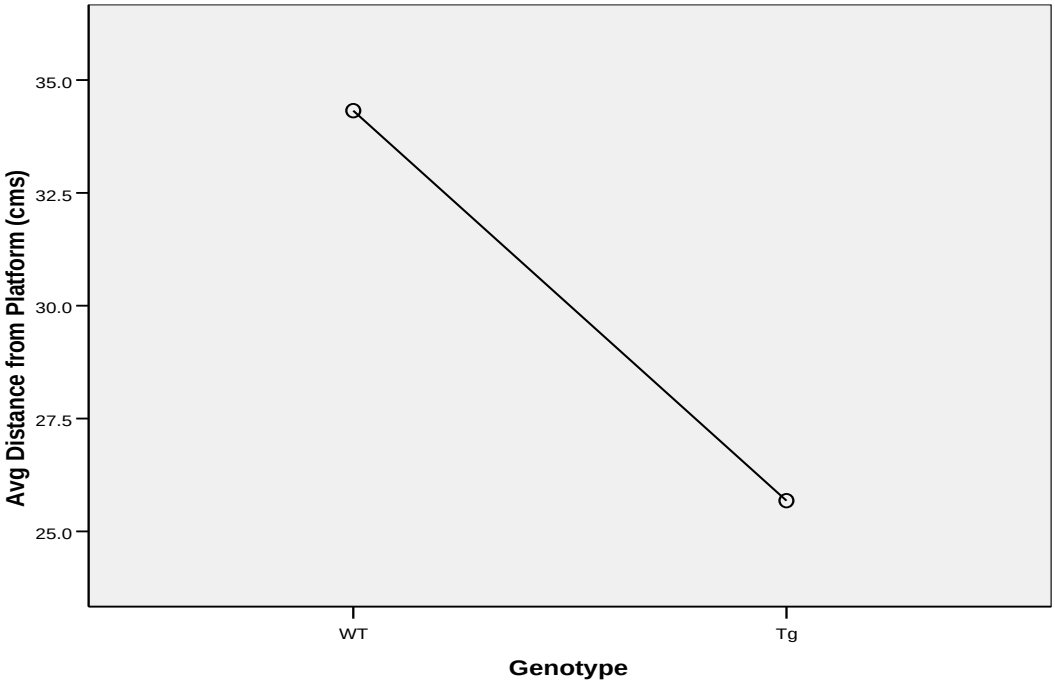


Figure 4: Probe trial Gallagher measure (Avg. distance from platform in cms) Main effect of Genotype

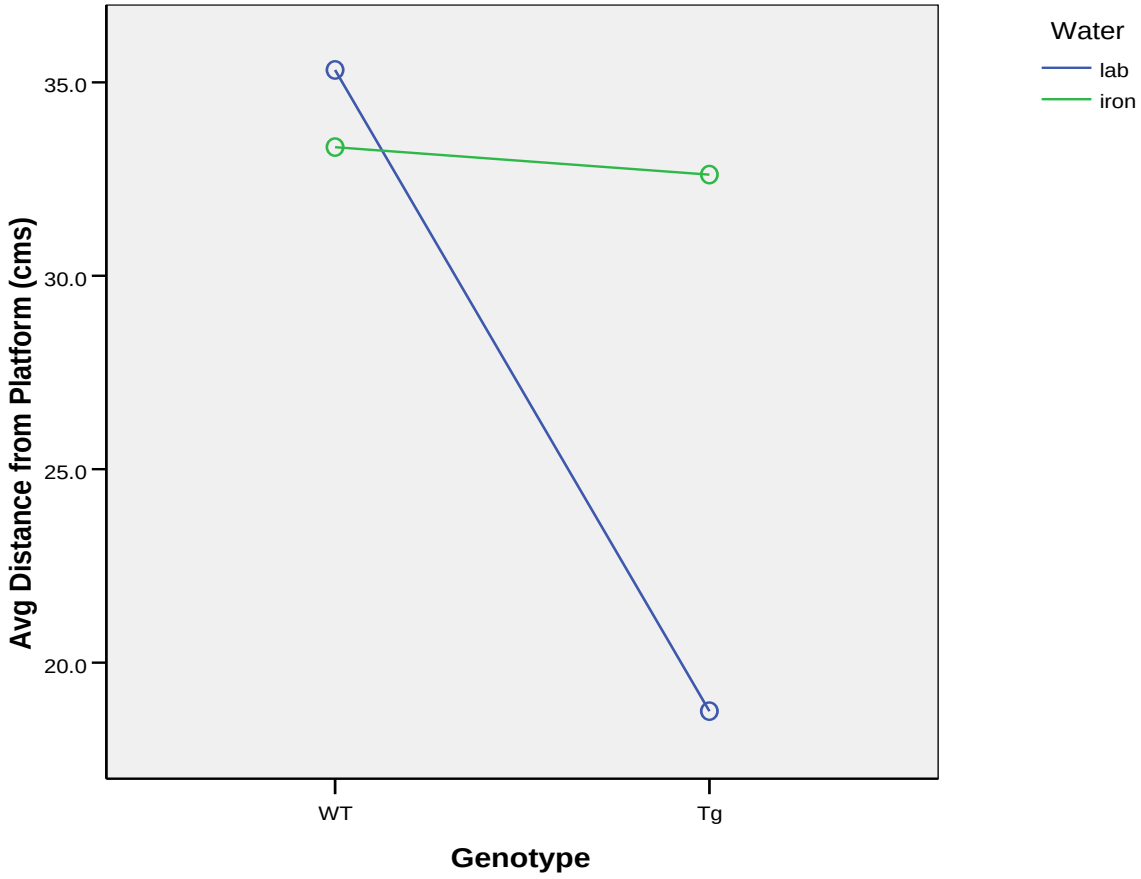


Figure 5: Probe trial Gallagher measure (Avg. distance from platform in cm)
Trend for interaction of Genotype X Water

Table 3: Means and standard deviations for Moving Platform Escape Latencies (seconds) Day 1 (trials a, b, c, & d)

	Genotype	Water	Mean	Std. Deviation	N
1a	Wt	Lab	36.88	22.36	9
		Iron	30.00	19.46	9
	Tg	Lab	49.77	12.97	9
		Iron	38.71	23.11	7
1b	Wt	Lab	39.77	20.67	9
		Iron	28.88	22.59	9
	Tg	Lab	55.22	14.33	9
		Iron	46.14	16.04	7
1c	Wt	Lab	48.88	14.45	9
		Iron	27.77	25.76	9
	Tg	Lab	44.00	17.23	9
		Iron	54.28	11.64	7
1d	Wt	Lab	56.55	6.83	9
		Iron	37.77	23.02	9
	Tg	Lab	51.33	13.15	9
		Iron	46.28	17.37	7

Table 4: Means and standard deviations for Moving Platform Escape Latencies (seconds) Day 2 (trials a, b, c, & d)

	Genotype	Water	Mean	Std. Deviation	N
2a	Wt	Lab	24.55	16.41	9
		Iron	33.33	24.31	9
	Tg	Lab	49.77	14.87	9
		Iron	26.57	17.39	7
2b	Wt	Lab	42.11	15.67	9
		Iron	37.11	19.21	9
	Tg	Lab	46.88	16.48	9
		Iron	50.00	16.21	7
2c	Wt	Lab	51.52	11.51	9
		Iron	31.03	20.59	9
	Tg	Lab	51.81	17.99	9
		Iron	47.05	15.32	7
2d	Wt	Lab	33.33	17.16	9
		Iron	41.88	20.09	9
	Tg	Lab	54.55	16.33	9
		Iron	36.85	18.84	7

Table 5: Means and standard deviations for Moving Platform Escape Latencies (seconds) Day 3 (trials a, b, c, & d)

	Genotype	Water	Mean	Std. Deviation	N
3a	Wt	Lab	50.44	15.13	9
		Iron	37.22	22.30	9
	Tg	Lab	49.77	18.72	9
		Iron	25.71	24.11	7
3b	Wt	Lab	41.11	23.19	9
		Iron	44.88	20.97	9
	Tg	Lab	42.55	22.36	9
		Iron	33.14	25.88	7
3c	Wt	Lab	38.77	22.63	9
		Iron	42.11	21.70	9
	Tg	Lab	47.22	25.35	9
		Iron	27.28	21.97	7
3d	Wt	Lab	42.77	20.49	9
		Iron	31.11	22.15	9
	Tg	Lab	53.33	13.26	9
		Iron	24.14	24.80	7

Table 6: Means and standard deviations for Moving Platform Escape Latencies (seconds) Day 4 (trials a, b, c, & d)

	Genotype	Water	Mean	Std. Deviation	N
4a	Wt	Lab	45.77	19.81	9
		Iron	39.33	25.33	9
	Tg	Lab	39.77	24.67	9
		Iron	29.71	22.54	7
4b	Wt	Lab	35.22	25.40	9
		Iron	40.44	18.24	9
	Tg	Lab	37.22	25.21	9
		Iron	46.14	13.50	7
4c	Wt	Lab	42.11	19.24	9
		Iron	33.71	21.25	9
	Tg	Lab	50.21	17.16	9
		Iron	33.90	24.66	7
4d	Wt	Lab	40.22	22.19	9
		Iron	38.88	25.72	9
	Tg	Lab	45.77	23.57	9
		Iron	32.14	17.90	7

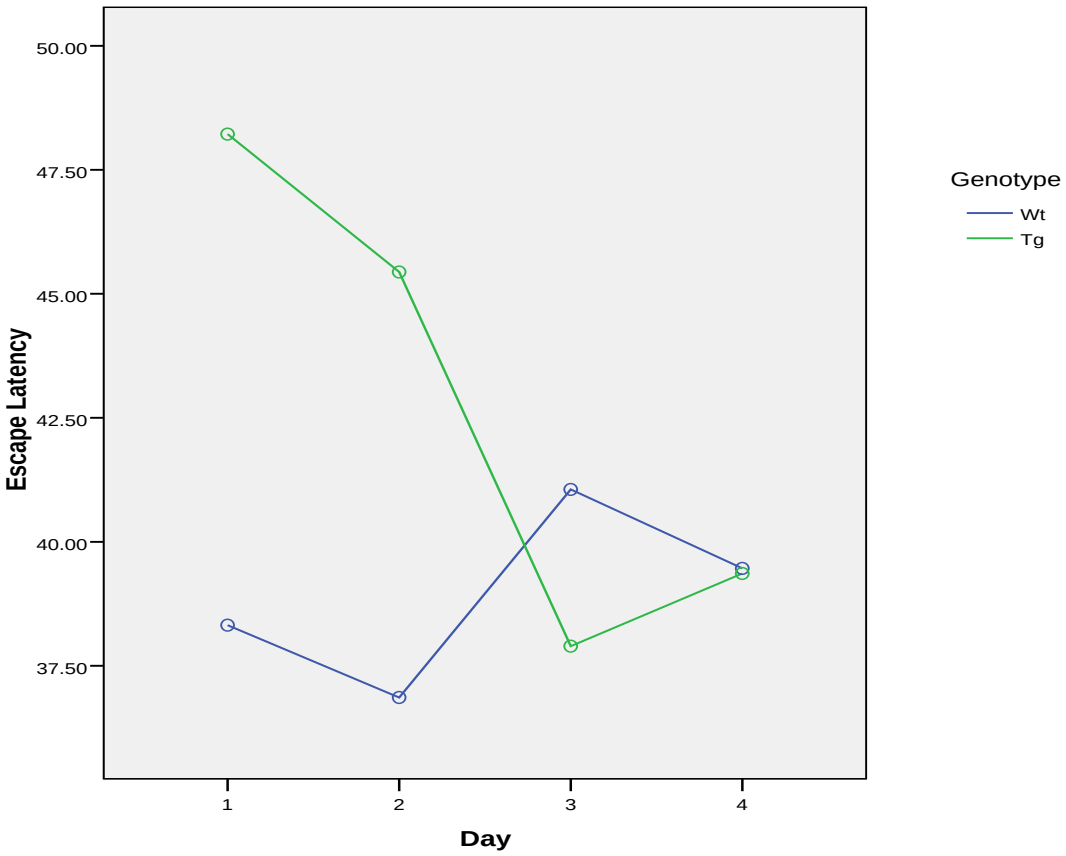


Figure 6: Interaction of Day X Genotype for Moving Platform Escape Latencies (in seconds)

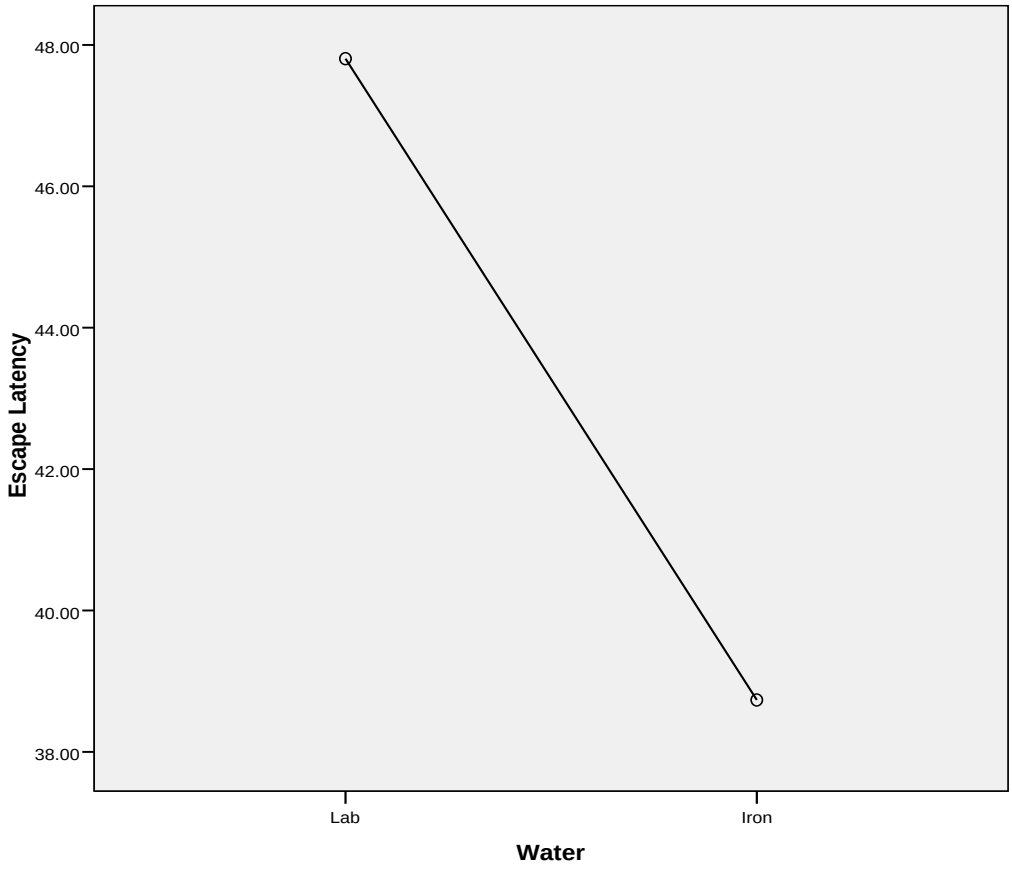


Figure 7: Main effect of Water for the Moving Platform Escape Latencies (in seconds)

Table 7: Means and standard deviations for Plaque Area (pixel values) for Congo red Measurements
(Regions 1-5 Followed by Birefringent Regions 1b-5b)

	Water	Mean	Std. Deviation	N
roi 1	Lab	108.59	61.98	9
	Iron	123.87	55.56	7
roi 2	Lab	126.82	47.97	9
	Iron	115.93	54.53	7
roi 3	Lab	147.81	9.70	9
	Iron	106.39	73.43	7
roi 4	Lab	125.60	47.95	9
	Iron	60.28	75.23	7
roi 5	Lab	96.88	66.16	9
	Iron	58.18	74.82	7
roi 1b	Lab	54.90	46.03	9
	Iron	62.11	63.26	7
roi 2b	Lab	48.77	47.55	9
	Iron	66.77	66.40	7
roi 3b	Lab	66.61	49.06	9
	Iron	26.49	35.90	7
roi 4b	Lab	45.62	40.51	9
	Iron	16.48	29.96	7
roi 5b	Lab	29.15	30.36	9
	Iron	22.65	31.71	7

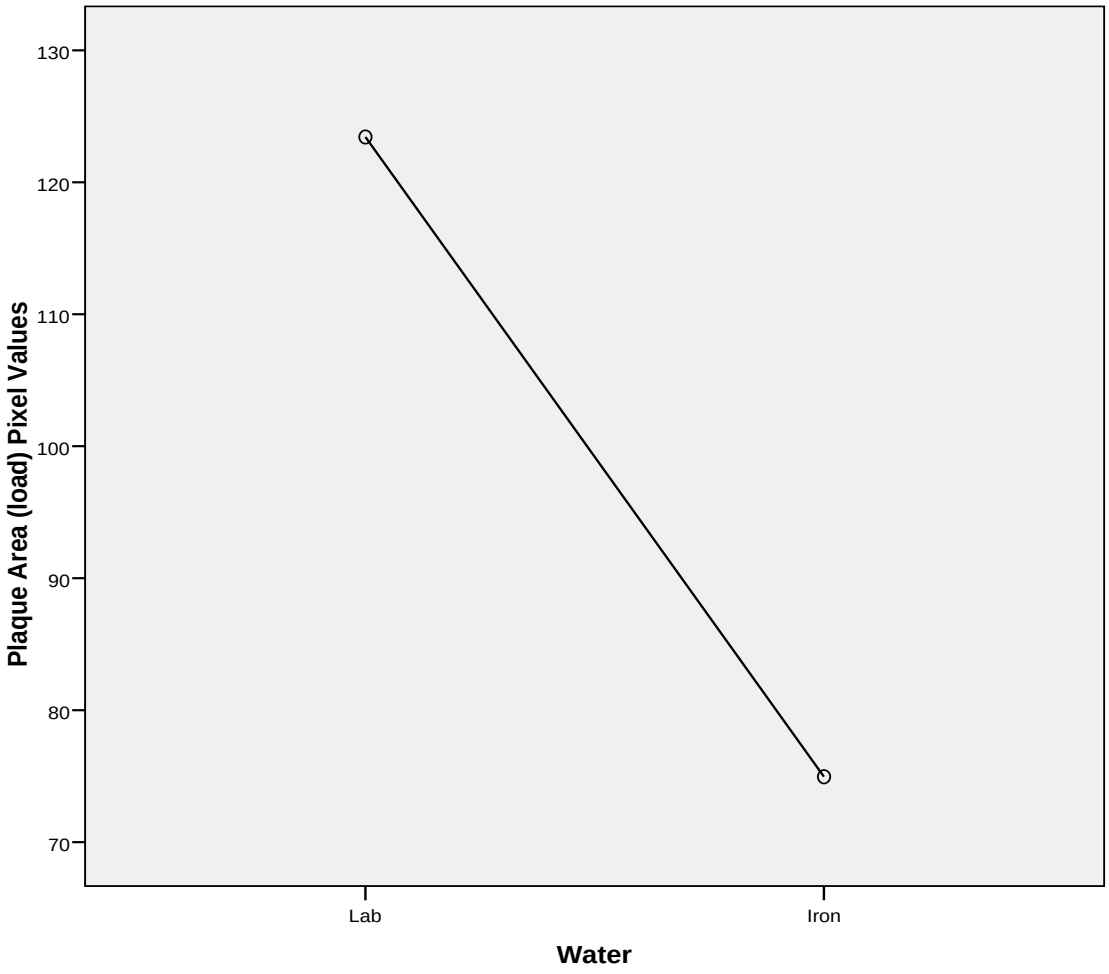


Figure 8: Main effect of Water for Congo Red Plaque Areas (pixel values)
Regions 3, 4, & 5

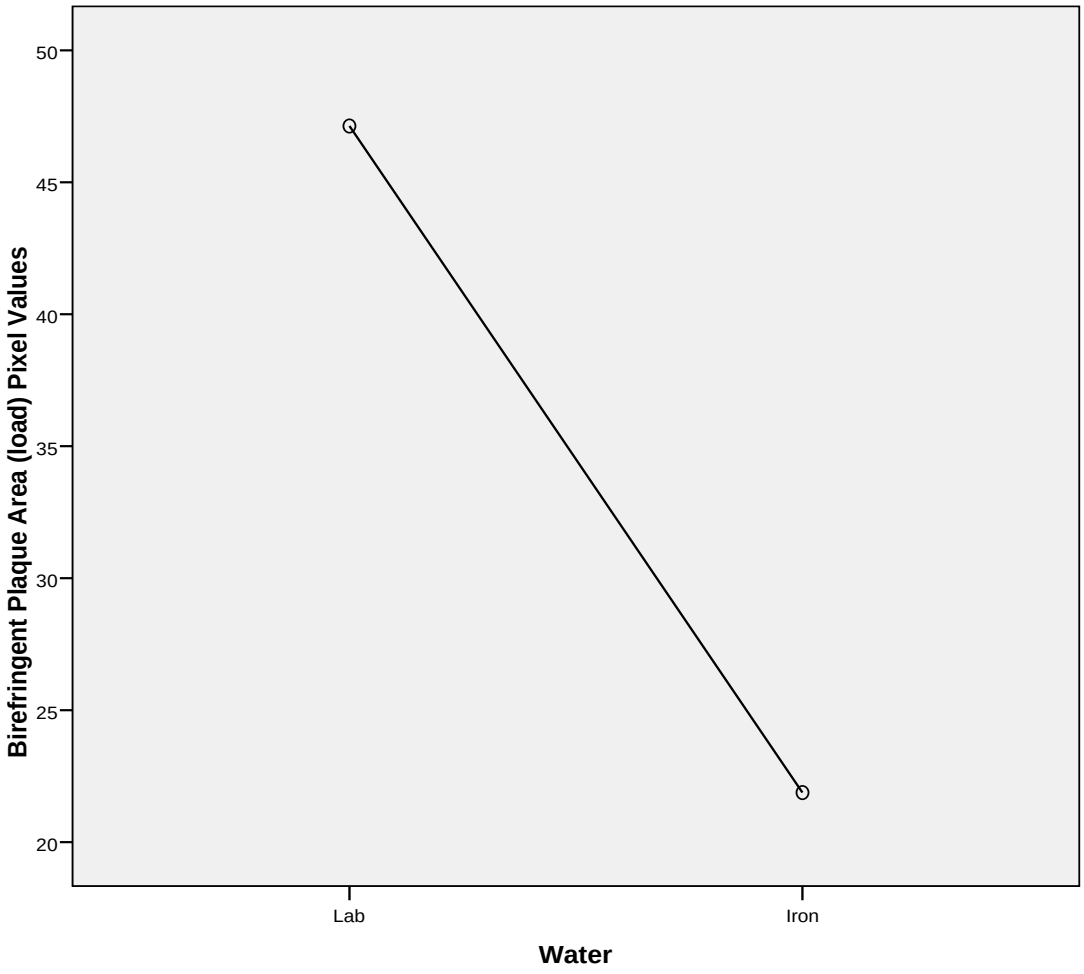


Figure 9: Trend of Main Effect of Water for Congo Red Birefringent Plaque Areas (β-pleated Sheets) for Regions 3, 4, & 5

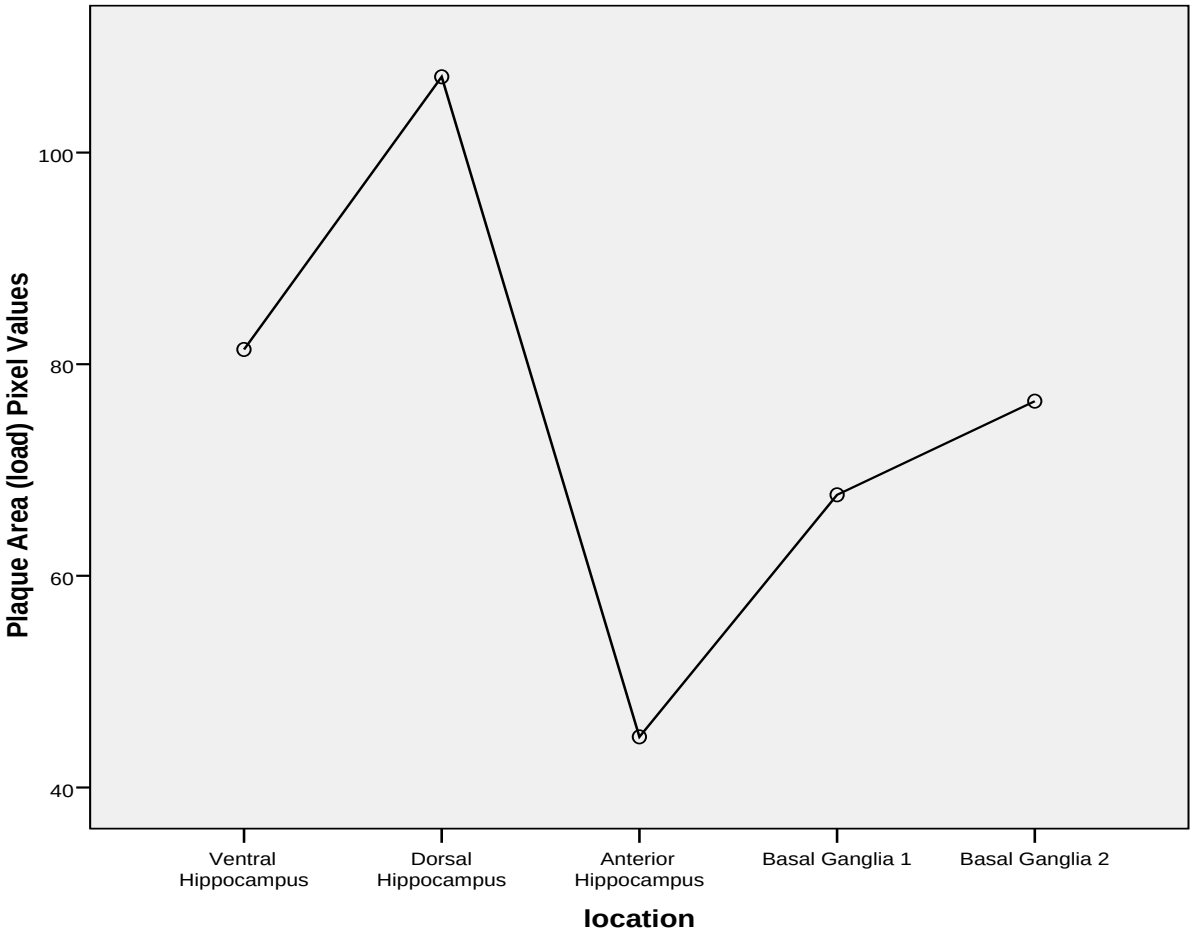


Figure 10: Main effect of Location for All Plaque Areas
Non-Birefringent and Birefringent Combined

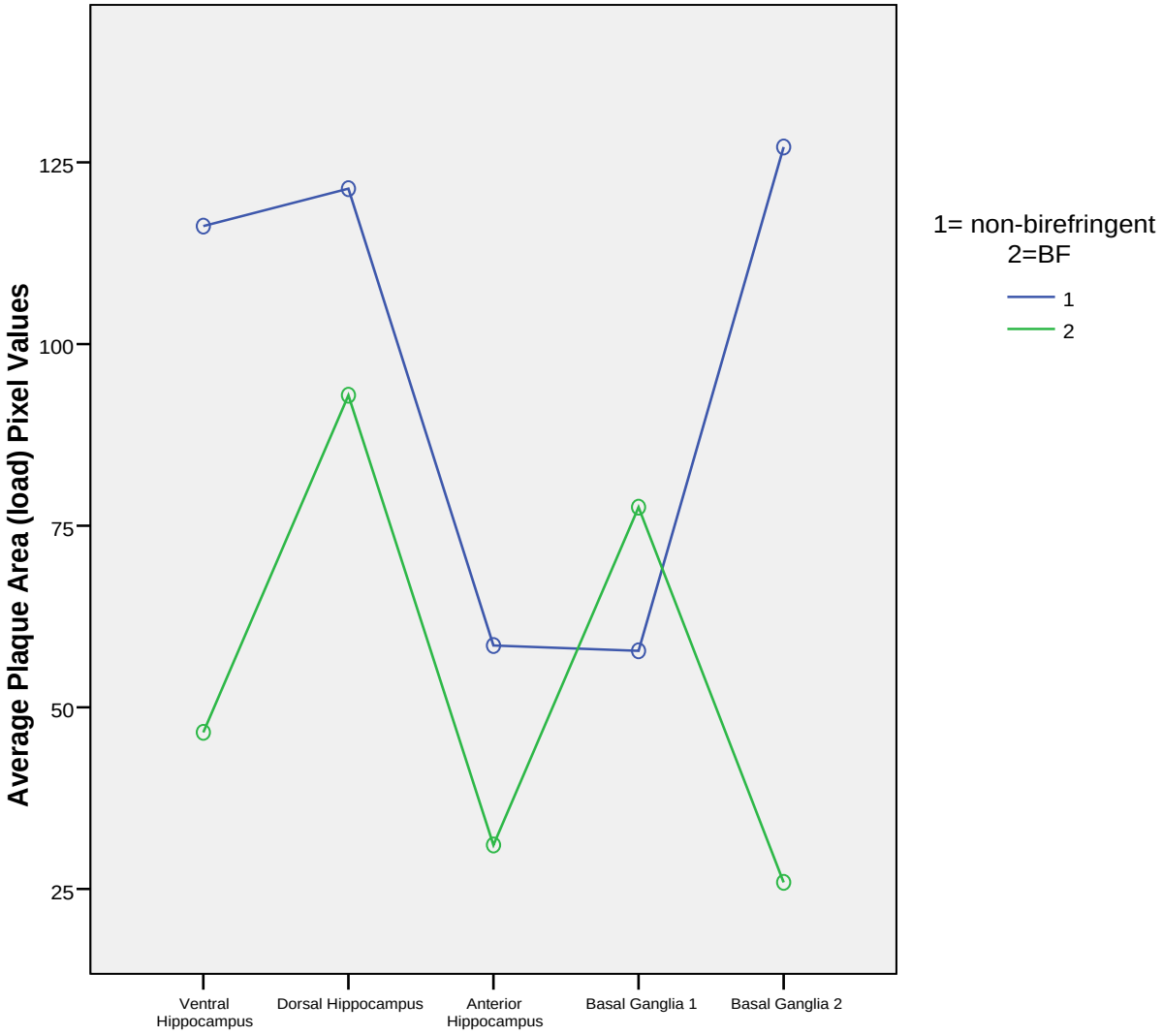


Figure 11: Main effect of Location for Congo Red Plaque Areas by Type (Type 1: Plaque Area; Type 2: Birefringent Plaque Area)

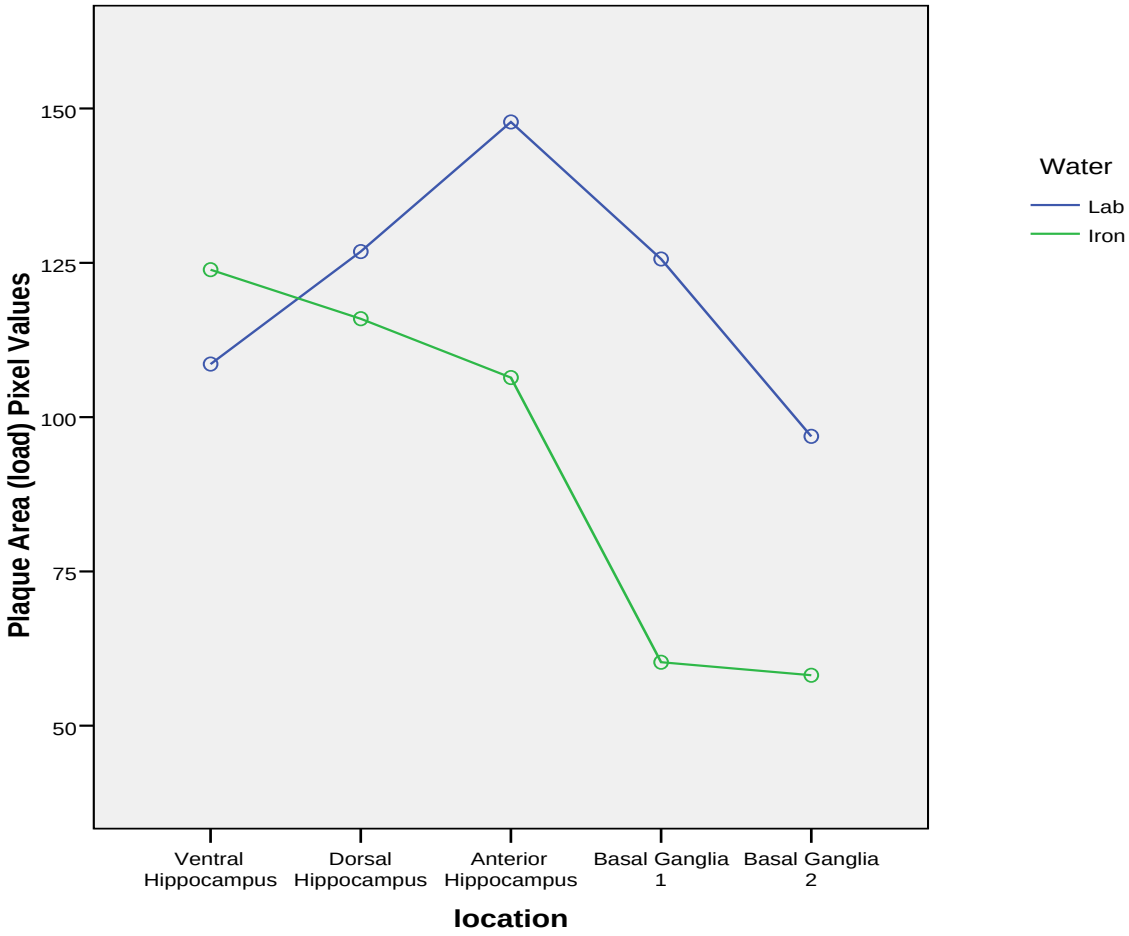


Figure 12: Main effect of Location for Congo Red by Water Type
For Non-Birefringent Plaque Areas (Pixel Values)

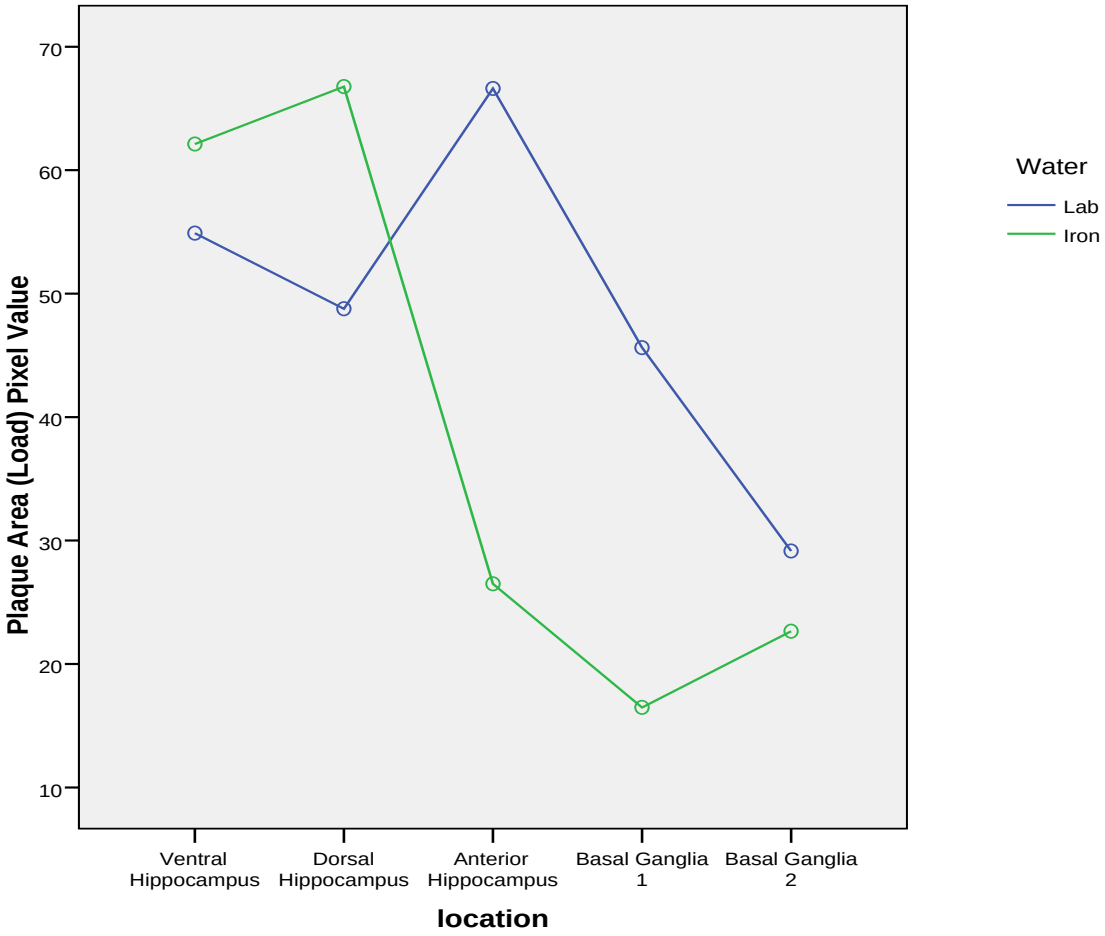


Figure 13: Main Effect of Location of Plaque Area for Congo Red by Water Type Birefringent Plaque Areas (Pixel Values)

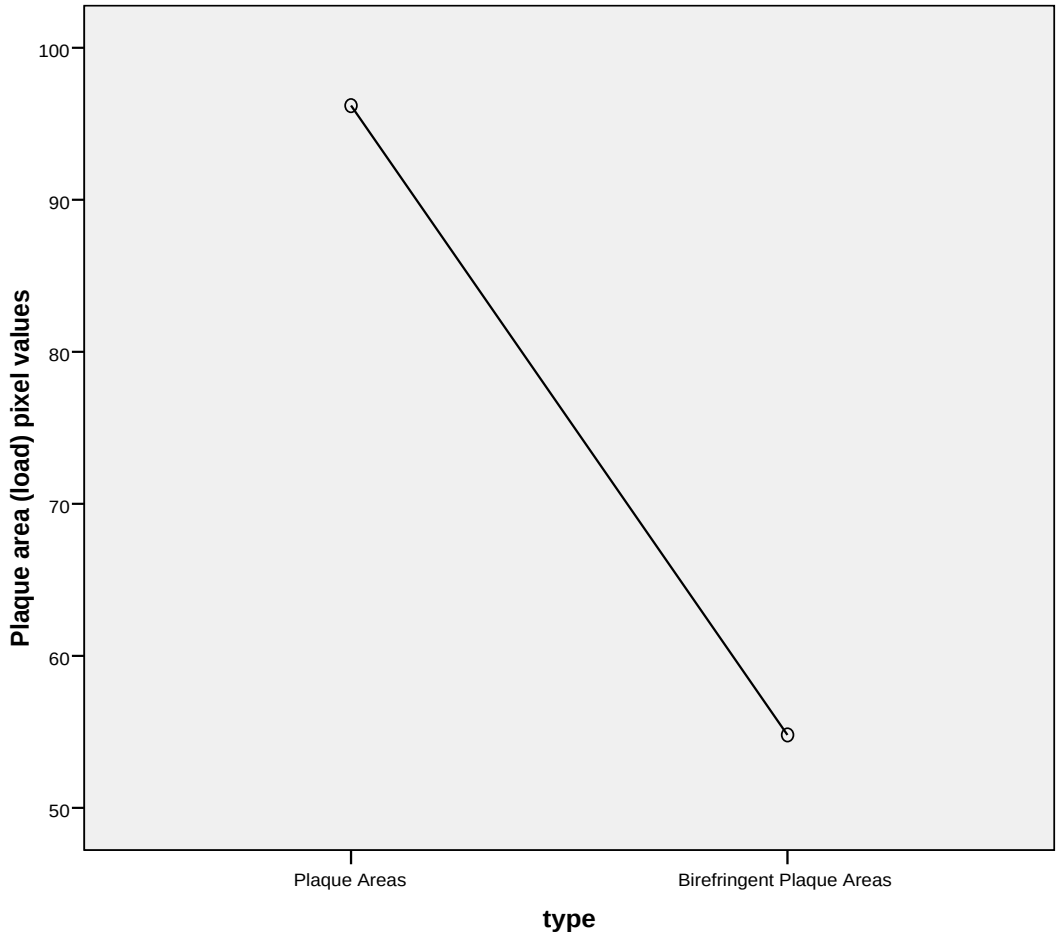


Figure 14: Main effect of Plaque Type for Congo Red Plaque Areas

4. Discussion

Studies such as this are important because there is no current cure for AD, and biologically based behavioral studies help to give more complete pictures about diseases of the CNS, and their possible causes and treatments. This is because there are two components to AD: the biological changes and the behavioral manifestations that are the result. It was hypothesized that animals in the dietary enhancement of $FeNO_3$ groups (Wt and Tg) would perform worse in the MWM than mice in the lab tap water groups (Wt and Tg).

However, this was refuted by the actual data. There was no difference between Fe Wt and Tg groups. In contrast lab Wt out performed lab Tg. The initial analysis of the Atlantis paradigm A & B trials for latency yielded no significant results. This is probably because the task was simply too difficult for the Tg mice to successfully master (However, this could be due to a small N, or because of large SD's). Although, the Wt mice did show improvement in escape latency scores across days (refer to appendix pp. 62-63), which does indicate learning for these groups. Probe trials, in contrast, yielded many interesting results, as did the histological data, with both measures revealing some surprising and new findings.

Behavioral Data

Percent in quadrant data for probe trials demonstrated that Fe Tg animals unexpectedly spent more time in the correct quadrant than lab Tg animals, and lab Wt spent more time in the correct quadrant than lab Tg as expected. This suggests that the dietary enhancement of Fe had a neuroprotective effect in the Tg animals; this is a new finding. This could be due to the fact that animals were administered Fe prenatally and could have developed a more efficient Fe processing system. Additionally, it could mean that the Fe levels administered in the water did not have an overload effect as expected, but was more of a supplement that enhanced function and behavior. Lastly, it could be a reverse effect where persistent animals (mice with HC damage) continue searching for the platform while animals with no such HC deficits move on.

The probe trial Gallagher measures also gave some surprising results with Tg animals apparently swimming closer to the platform than Wt animals. This is a hard result to interpret, since it is the exact opposite of what you would expect. Further examination of the swim patterns and quadrant location revealed that this measure was highly inaccurate. Animals that actively swam around the pool looking for the platform were given poor scores, while animals that swam in tight circles in the improper quadrant were given good measures. For examples of these swim patterns refer to the appendix (pp. 68-71). As, a result it is being concluded that the Gallagher measure is not a good measure for Tg2576 mice, and it is suggested that swim patterns be examined before making any conclusive statements about this behavioral measure.

However, as stated before, this result could be due to persistence, not poor spatial memory. There could also be contributing factors coming from variables that have not been analyzed like passive (no movement) and active (stop and start movements) floating (a common behavior observed at GMU in Tg animals) or circling (also a commonly observed Tg behavior). Therefore, animals that float near the platform would be counted as closer while animals actively exploring for the platform may achieve higher scores and appear to have HC deficits.

For the moving platform, Wt had faster escape latencies than Tg on days 1 & 2 and Fe groups did better than animals raised on lab water. These results are surprising as it was expected that Fe groups would perform worse than lab groups. These results, once again point to a neuroprotective effect that iron may have had in Fe Tg animals. Perhaps, this effect was due to prenatal exposure and a more efficient Fe processing system within the brain and body. The other explanation could be that these animals were so well trained at the task at this point in the experiment that they were no longer motivated to find the platform and continued to explore.

Histology

The histological analysis revealed that lab Tg animals had more plaque areas than Fe Tg animals, particularly in regions 3, 4, and 5. This is the opposite of what was hypothesized, but the results match the behavior because the lab Tg group (with the most plaque areas) consistently was the worst performing group in the MWM. This suggests, that the more plaques in the brain, the more decline is seen in memory systems (i.e. poor performance in the MWM). Another point is that this study included two regions of the

HC that are not typically analyzed in the literature; Region 1 (ventral HC) and Region 3 (anterior HC). The majority of histology is done on the dorsal part of the HC (Region 2).

The inclusion of Region 1 (ventral hippocampus) and Region 3 (anterior hippocampus) was done in an effort to provide more information about the progression of the disease by including regions of the brain that are not typically analyzed. As Fe is most abundant in the BG, in adults, it was expected that Regions 3, 4, and 5 would have the most differences in plaque area due to the treatment of Fe in the drinking water. This was true from a statistical standpoint, but it is noteworthy to point out that mean scores of Fe Tg animals showed the highest plaque averages in Region 1 (ventral HC). Lab Tg demonstrated the highest plaque averages in Region 2 (dorsal HC) (refer to Table 7).

However, due to a small population and large SD's the statistical significance was only found in Regions 3, 4, and 5; with Region 3 (anterior HC) having the largest plaque areas and lab Tg having more plaque areas than Fe Tg. This has never been reported before and the results from this group of mice (cohort 3) should be compared with future groups (cohorts 4 and 5) to see if this pattern is unique to this study or if the data is similar. In addition, there were more plaque areas than birefringent plaque areas. This may be due to the age of the animals. Results from this study should also be compared to future studies of animals with a more advanced age (cohort 4, cohort 5) to see if birefringent plaques increase with the age of animals and further progression of AD as would be expected.

Limitations

Perhaps one of the most obvious limitations is that to our knowledge, no other behavioral work involving Fe as a treatment and animal models of AD has been performed, except here at GMU. Therefore, we had no guidelines to follow except our own ideas and preliminary studies done in our lab with rats and then Tg mice. Even though we did get good data with previous experiments, it is not apparently obvious that the same results can be achieved with mice as with rats for a few reasons. For one, mice have entirely different observed behavioral characteristics than rats; they do not behave the same socially, and display different learning characteristics. They also might have different metabolites in response to the dietary enhancement of the different water treatments; what may be enhancement to rats may be supplementation to mice and vice versa.

In addition, animals were bred at GMU, 4 Tg males and 16 Wt females. It was expected that 25% of the offspring would carry the APP mutation. However, this was not the case and so there were less Fe Tg animals than Lab Tg animals. Furthermore, lab animals had more successful birth rates than Fe animals. Lab water treated moms had more pups that Fe water treated moms and therefore more animals were in the lab water groups. This created the low population and since the treatment was prenatal, lab and Fe groups had already been established a priori. Therefore, they could not be changed to accommodate the differences in animal numbers. Future studies should genotype animals as young as possible, have more breeding pairs, and re-breed animals accordingly to adjust for possible low birth rates between groups. This would ensure a higher

population of experimental animals and provide more significant results due to higher similarities between animal groups.

Age of Testing

Secondly, it is important to note that animals began testing at a relatively young age for this particular genetic model. The animals in the study were tested at 12 months of age. Plaque deposition begins at 9 months of age in the Tg2576 animals, and this may not be relevant to advanced stages of AD. While this may not create a problem when performing histological studies it becomes extremely relevant in studies involving behavior. This could be because in humans the neurodegeneration occurs for years (which translates into months for mice) before any behavioral deficits, such as noticeable memory impairment begins to occur. A possible conclusion is that the more aged the animals become, the more representative the model becomes to AD research. Future studies should employ a more advanced age requirement prior to the implementation of any behavioral measures. However, significant differences were seen between Wt and Tg mice.

The Morris Water Maze: Confounds

In terms of the MWM it became evident by the present study (cohort 3) that this particular behavioral measure may not be the best choice for Tg2576 mice. This is for several reasons, the size of the pool was no longer in question, and due to previous studies with both 5' and 3' pools, the 4' pool was the best choice. In addition, this is a neurodegenerative and geriatric model, the mice are not only suffering from a progressive CNS disorder they are also aged. This is a problem because proper testing of the Tg2576

requires advanced age. The Atlantis paradigm is taxing physically (due to the demand on motor function) and might be too difficult mentally (due to the demand on perceptual ability) for this level of impairment. Traditionally the MWM has been designed to test both spatial (HC) and non-spatial (BG) forms of memory using different types of platforms (hidden vs. visible) (Morris, Garrud, Rawlins, & O'Keefe, 1982; Bannerman et al., 1995).

Therefore, the order of the tests administered might greatly affect the outcome of the results. For example, it is accepted in the literature that after the hidden platform (spatial task) is completed then a visible platform (non-spatial) task follows (Cain, 1998; Gerlai & Clayton, 1999; Gerlai & Roder, 1996). Essentially, you are first measuring HC function and then following with a task that measures general behavior performance abilities (Wolfer, Muller, Stagliar, & Lipp, 1997). This could present problems because you first present experimentally naïve animals with a difficult spatial task (hidden platform) and progress to a much easier and non-spatial task (visible platform). Therefore, the differences in animal performances between one task and the other could be due to different levels of habituation and procedural learning that have occurred between one task and the other. It was found, that mice swim in circular patterns around the edge of the pool, as a strategy to find the large platform, which yields poor results for the Gallagher measure.

As a result, the information that statistical analyses will provide can lead to false conclusions about HC function. In reality, the changes seen in behavior might be due to habituation and procedural learning, not HC function (Gerlai, 2001). So it would be

relevant to say that this particular design might not be ideal for assessing HC deficits in Tg2576 mice. Some alternate choices would be to either reverse the order of the platforms or run experimentally naïve animals (requires a larger N) in both tasks. This would ensure that the difficulty of the tasks is the same for both experimental designs. In addition, one could possibly eliminate latency trials and focus solely on probe trials and put more emphasis on platform seeking behaviors like percent in quadrant, platform crossings, and Thigmotaxicity. These measures are more sensitive to spatial learning deficits than escape latency, because they show a preference for the target location (Gallagher, Burwell, & Burchinall, 1993).

The other problem is that the timing of the probe trial itself might contain a large confound. This is because the probe trial comes at the end and it is a long trial (60 secs) in comparison to other trials that by the end of the experiment begin to shorten to lengths of a few seconds as animals learn the task. So the probe trial and the results associated might be linked more with an animal's persistence (which can indicate behavioral inflexibility; the inability to learn anymore) rather than preference for the platform location. Research has shown persistence to be a sign of HC dysfunction (Gerlai & Roder, 1996); animals with good HC function might actually give up looking for the platform in the target quadrant once they have discovered it is not there. This would yield a result of poor spatial memory performance in animals with no HC deficit, and good spatial performance scores in animals *with* HC deficits. One suggestion might be to either shorten the probe trial or to run repeated probe trials in between training trials

throughout the experiment. These suggestions could hopefully help to eliminate making obvious errors in future MWM testing in additional cohorts.

However, when the next generation of animals related to this study (cohort 4) was tested using a different method, which utilized a stationary platform with more trials in a day without probe trials, the task seemed to produce better results. Future studies should adopt this new method (Billings, 2005) and utilize a battery of tests (novel object recognition, fear conditioning, etc.) to give the best insight to behavior deficits, since one measure may not be enough to make assumptions about the behavior of particular models. For example, in preliminary MWM runs, the animals exhibited pathological patterns of circular behavior, so that it was impossible to tell if they knew where the platform was or not. No conclusions could be made about this behavior and it was not clear if the circling was caused by the treatment or if it was just a characteristic of this particular mouse model (Tg2576) of AD.

Enriched Environment

In addition, animals were all given access to exercise wheels, and the behavioral task chosen requires a fairly high activity level. Exercise has proven to generate new brain cells (van Praag, Shubert, Zhao, & Gage, 2005), and therefore the task could have actually improved memory while we were using it to assess memory impairments. Since the wheel running behavior was not documented, there is no data to determine if each mouse ran equally. Therefore, there is no way to determine what the impact wheel running had on the outcome of the data between animal groups or water groups.

However, it should not be ruled out, because exercise induced neurogenesis within the hippocampus is a well-documented and researched phenomenon (van Praag et al., 2005).

Water Confounds

In the beginning of the study breeder females were treated with 10ppm $FeCl_3$ in the drinking water, which continuously precipitated (separated from the water and clung to the sides of the water bottle) and had to be shaken several times of day. Then buffer was added to the water to keep the $FeCl_3$ from precipitating out of the water. $FeNO_3$ was then used and after much trial and error did yield a successful result. However, all of this could have had an impact on the effectiveness of the treatment by fluctuating iron levels in the water. This could be a result of starting the pregnant moms on Iron Chloride enhanced water, and then later Iron Nitrate enhanced water or even the change in the pH of the water by adding a buffer. The upside is that this information was put directly into effect with cohort 4 animals, so the water problem had been resolved by the start of the new study. The question that remains is did we enhance iron levels to a point of overload or did we simply supplement the animals? The results of this study suggest that a higher level of iron might be used to avoid possible supplementation and more directly model overload of iron.

Tg2576 Confounds

Even though these transgenic models of human CNS diseases are extremely important they do not come without drawbacks. Some of the problems found in this line of research here at GMU are that white mice with red eyes have vision problems. If the

right lighting is not used, the animals cannot find the platform as easily as the other animals as discovered in the first generation of animals at GMU (cohort 1). Secondly, if the water temperature is too warm, we used 22-24° C, animals do not rush to find the platform (as is the point of the task); instead, they explore the pool and do not go to the platform until they become tired.

Also, the genotyping itself proved not to be an easy task. Three separate runs were done here at GMU with inconsistent results, and then tails were sent to Taconic for comparison results. There were some differences in the GMU runs and the Taconic run. It is noteworthy to point out those primers and solutions previously used for genotyping had been stored at -80° C for over a year, and new materials had to be ordered. The new materials produced results, but they were not always conclusive or obvious to determine. This is why tails were finally sent to Taconic for a professional analysis. However, histological analysis did reveal plaques in all animals reported as Tg by Taconic, which were the results, used.

Blindness

After the completion of this study, it was discovered that the Tg2576 mice carry a recessive gene for blindness (~10% frequency). As a result, swim paths were examined for pathological differences and visible platform days were examined to see if any obvious mean differences were apparent. Visible platform swim patterns revealed that several mice with 60-second latencies (the duration of the trial) did in fact find the platform, but continued on. When examining both days of visible only one mouse was found to have not found the platform at all (4 trials). This animal was then dropped from

the analysis to see if significance was altered. All measures remained significant, but to ensure complete accuracy this will be examined in great detail to determine if the results reported here portray an accurate picture. However, it is impossible to know without genetic testing whether or not these animals did in fact carry the blindness gene. This will be done in a separate study, and results will be reported accordingly. Future generations of similar studies (cohort 4) will utilize this information and analyze the results accordingly.

A preliminary analysis of a later group (cohort 4) has shown that mice that did find the visible platform with a long latency, but could not use the distal cues to find the submerged platform, were shown by genetic testing to be blind. Animals from the present study, that demonstrate this behavioral pattern, will be analyzed by genetic analysis of tail snips if possible. The data will then be reexamined accordingly. It is possible that the poor performance of the lab Tg mice could be due to blindness.

Conclusion

The results obtained did not support the original hypotheses, but the dietary enhancement of Fe did have an effect on this AD model. This was demonstrated, by Fe Tg animals demonstrating a neuroprotective effect, and having fewer plaques than Lab Tg animals. A different effect was seen in the outcome of Fe treated breeder mice litters, since there were fewer pups in the Fe treated groups for both Tg and Wt, than in the lab treated groups. This could mean that only the strongest animals survived and this could have affected the results. This leaves more room for variability in the health of lab water treated groups, which also would have impacted the outcome of the results. An

unresolved question would be why were Fe Tg groups so similar to Fe Wt and Lab Wt groups? A larger group of animals in future studies could amplify or explain further these results.

Results were not as we had expected for several reasons: for one the N was low, the SD's were very large, and there was little to no difference between Fe groups, and. All of these factors gave us results that were not very easy to interpret. However, Lab Wt mice consistently demonstrated significantly better scores than Lab Tg mice. Fe Tg and Lab Tg performed in a similar manner, suggesting that dietary enhancement of $FeNO_3$ in the drinking water had a treatment effect on Tg animals. The most interesting results were found in the histological analysis with Fe Tg having less plaque area than lab Tg; specifically in regions that are not typically analyzed. These new findings indicate that Fe does play a critical role in AD, and more behavioral research is needed to make any causative links between Fe and AD in Tg mouse models.

APPENDIX

Means and Standard Deviations for Atlantis platform escape latencies Days 1-4 (A&B trials)

	WATER	GENOTYPE	Mean	Std. Deviation	N
LAT1A	lab	Wt	51.3222	15.94599	9
		Tg	58.0444	4.84436	9
	iron	Wt	54.0000	11.91260	9
		Tg	53.9000	16.13908	7
LAT1B	lab	Wt	41.7333	19.42370	9
		Tg	58.2778	5.16667	9
	iron	Wt	54.1889	15.88462	9
		Tg	57.8143	5.78286	7
LAT2A	lab	Wt	50.4889	14.41696	9
		Tg	55.0444	14.86667	9
	iron	Wt	52.1667	14.17074	9
		Tg	56.0714	10.39402	7
LAT2B	lab	Wt	41.8778	22.20370	9
		Tg	54.0556	14.23860	9
	iron	Wt	33.0556	22.70760	9
		Tg	52.6857	12.72470	7
LAT3A	lab	Wt	38.4333	16.17367	9
		Tg	46.3111	14.74461	9
	iron	Wt	50.3111	16.40255	9
		Tg	48.8571	17.52901	7
LAT3B	lab	Wt	38.7222	23.06029	9
		Tg	37.1111	24.01398	9
	iron	Wt	31.5889	23.83005	9
		Tg	41.7286	18.90050	7
LAT4A	lab	Wt	37.3444	23.77873	9
		Tg	48.2444	18.81430	9
	iron	Wt	36.3667	23.25833	9
		Tg	40.3857	24.62319	7
LAT4B	lab	Wt	39.2889	21.13158	9
		Tg	46.2000	18.38634	9
	iron	Wt	37.3333	19.77909	9
		Tg	38.8714	23.15180	7

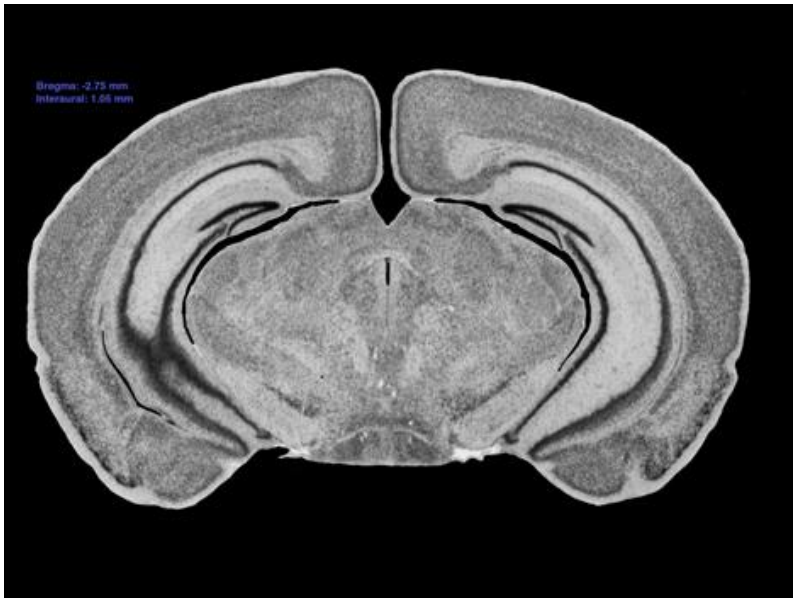
Means and Standard Deviations for Atlantis platform escape latencies Days 5-8 (A&B trials)

	WATER	GENOTYPE	Mean	Std. Deviation	N
LAT5A	**lab**	Wt	24.9444	21.73460	9
		Tg	43.1444	23.07228	9
	iron	Wt	43.6889	21.57432	9
		Tg	44.2429	26.93906	7
LAT5B	**lab**	Wt	32.2444	24.87464	9
		Tg	38.7889	27.26483	9
	iron	Wt	35.9667	20.69378	9
		Tg	47.0857	18.50805	7
LAT6A	**lab**	Wt	32.1667	20.52279	9
		Tg	46.7889	21.34306	9
	iron	Wt	33.1000	20.73210	9
		Tg	52.8000	19.04941	7
LAT6B	**lab**	Wt	32.2556	26.01221	9
		Tg	29.9333	24.08303	9
	iron	Wt	34.9222	23.79962	9
		Tg	52.3429	20.25890	7
LAT7A	**lab**	Wt	34.2556	21.75587	9
		Tg	43.9889	20.55033	9
	iron	Wt	29.7444	17.08911	9
		Tg	42.0571	22.83724	7
LAT7B	**lab**	Wt	35.2444	23.99198	9
		Tg	46.4556	13.39870	9
	iron	Wt	30.0444	21.82877	9
		Tg	35.1429	20.49852	7
LAT8A	**lab**	Wt	31.1333	21.09828	9
		Tg	41.9333	21.18000	9
	iron	Wt	32.0889	23.84866	9
		Tg	42.0571	24.09591	7
LAT8B	**lab**	Wt	31.3222	23.19482	9
		Tg	44.3778	23.65422	9
	iron	Wt	27.0222	21.87492	9
		Tg	33.2143	26.02572	7

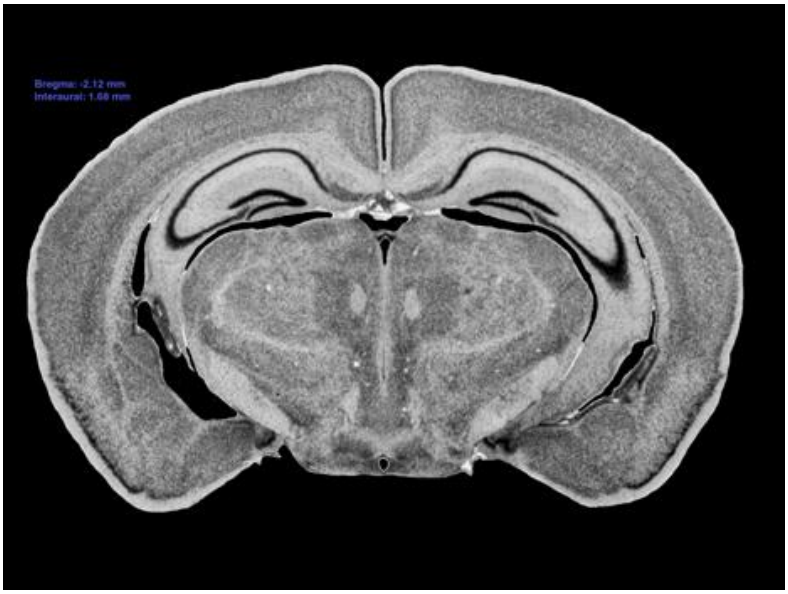
Visible Platform Means and Standard Deviations for Escape Latencies
Days 1 and 2

	Water	Genotype	Mean	Std. Deviation	N
Day1A	**lab**	wt	27.7444	17.74557	9
		Tg	42.9222	22.41851	9
	iron	wt	33.7889	23.30630	9
		Tg	43.3571	23.25452	7
Day1B	**lab**	wt	24.9444	21.24130	9
		Tg	48.8333	17.88952	9
	iron	wt	40.8889	24.34299	9
		Tg	41.6143	23.37894	7
Day2A	**lab**	wt	23.6556	23.89912	9
		Tg	42.8222	26.00855	9
	iron	wt	24.1111	20.33786	9
		Tg	23.9429	26.33039	7
Day2B	**lab**	wt	32.4556	24.69930	9
		Tg	44.6222	24.00322	9
	iron	wt	32.8778	26.35620	9
		Tg	31.7143	20.13301	7

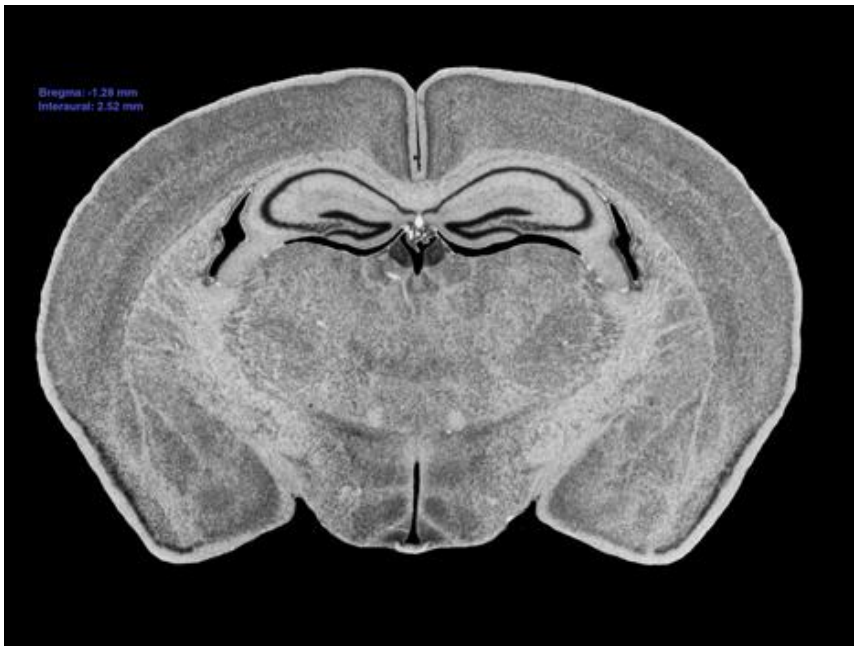
Histology Region of Interest Images (1-5)



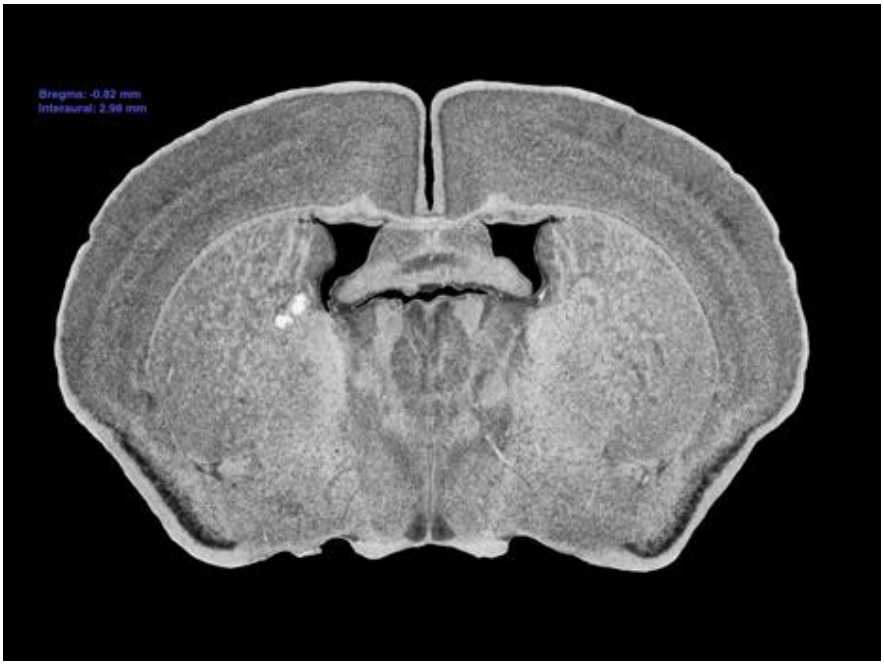
Region 1: Ventral Hippocampus



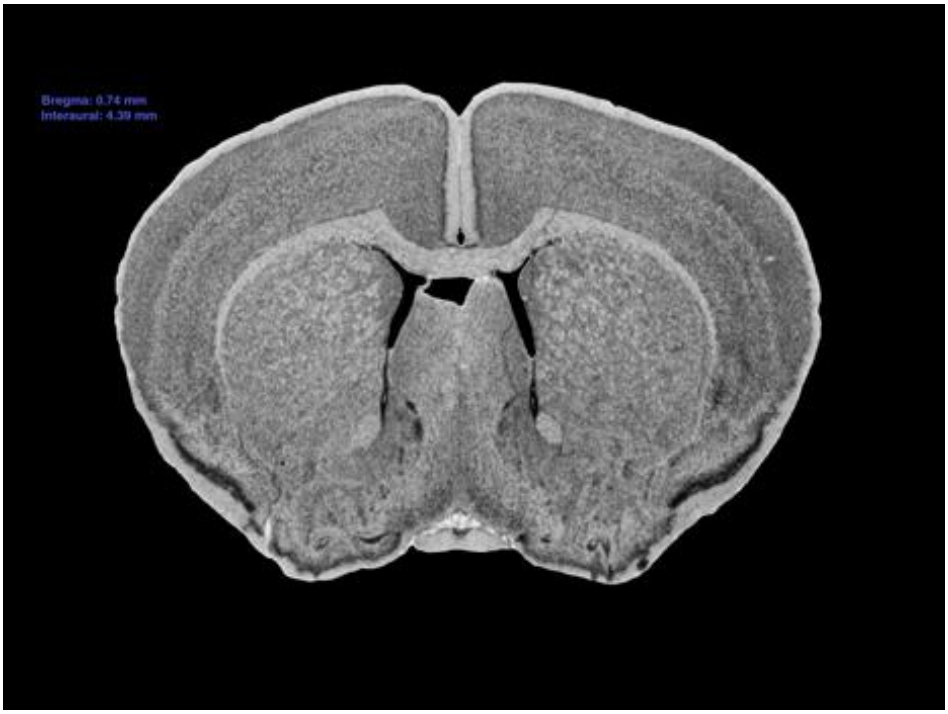
Region 2: Dorsal Hippocampus



Region 3: Anterior Hippocampus



Region 4: Basal Ganglia 1



Region 5: Basal Ganglia 2

Gallagher measure Swim Patterns:

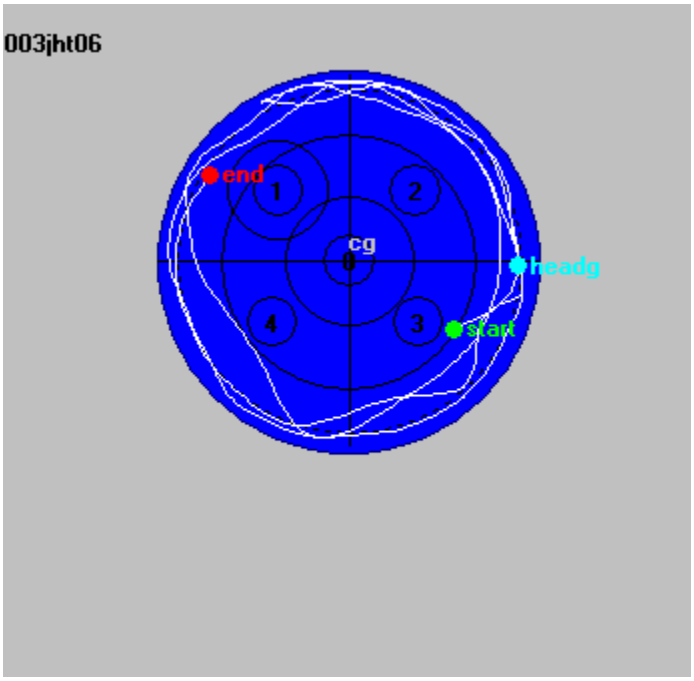
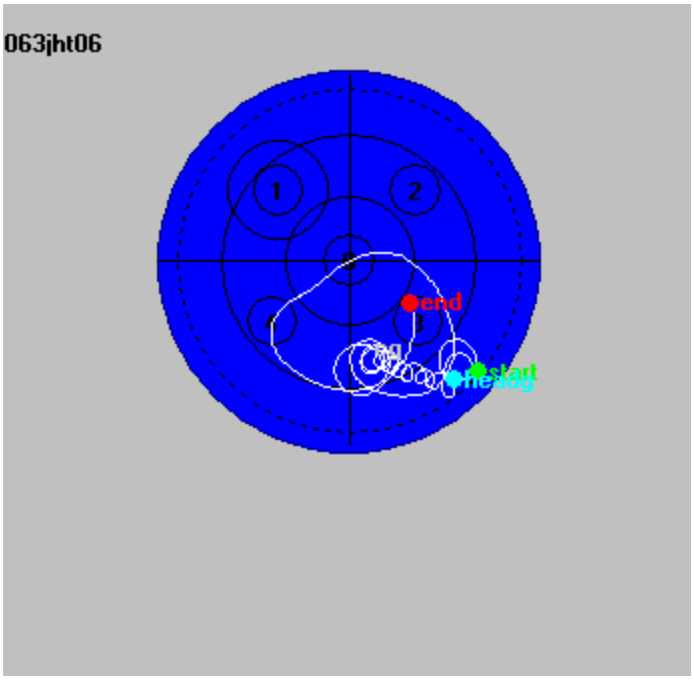


Figure 003 jht06: This image gives an example of what was recorded as a poor Gallagher measure; the animal does end the trial in the proper platform quadrant (quadrant 1). This was seen repeatedly in swim patterns where animals circled the pool looking for the platform; poor scores with relatively large amounts of time in the proper quadrant.



063 jht06: This figure gives an example of what was considered a good Gallagher measure; as you can see the animal never enters the correct quadrant (platform is in 1). Therefore, rendering the measure useless in this case, this animal spent zero time in the proper quadrant. This animal's swim pattern reveals no platform seeking behavior.

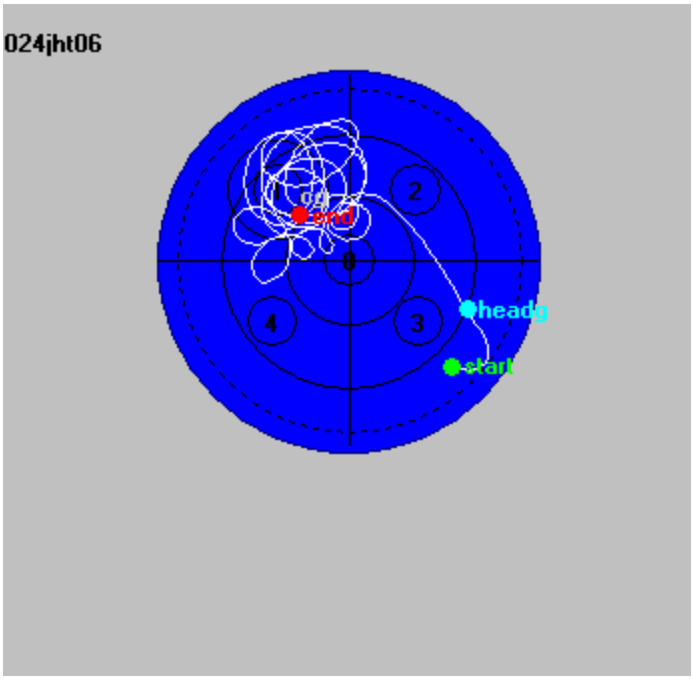


Figure 024jht06: This figure demonstrates a more accurate swim pattern of a good Gallagher measure. This animal spends the entire trial in close proximity to the platform.

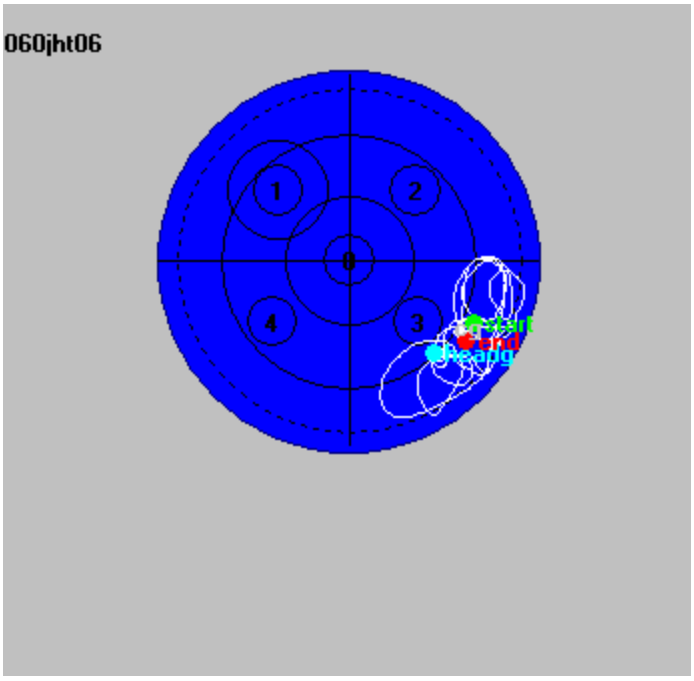


Figure 060jht06: This figure demonstrates an accurate swim pattern of a poor Gallagher measure. As seen above the animal spends no time in the proper quadrant and is not close to the platform quadrant at any point during the trial.

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