FEAR CONDITIONING AS A MEASURING TOOL FOR COGNITIVE DEFICITS RELATED TO AMYLOID BURDEN COUPLED WITH IRON, ZINC, AND COPPER IN THE TRANSGENIC TG2576 MOUSE MODEL FOR ALZHEIMER'S DISEASE

by

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Fear Conditioning as a Measuring Tool for Cognitive Deficits Related to Amyloid Burden Coupled with Iron, Zinc, and Copper in the Transgenic Tg2576 Mouse Model for Alzheimer's Disease

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List of Abbreviations

Aβ	beta amyloid
AD	Alzheimer's Disease
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APP	amyloid precursor protein
CS	conditioned stimulus
DV	dependent variable
GLM	general linear model
ICC	intraclass correlation coefficient
IGC	independent growth curve
IV	independent variable
Tg	transgenic
US	unconditioned stimulus
Wt	wildtype

Abstract

FEAR CONDITIONING AS A MEASURING TOOL FOR COGNITIVE DEFICITS RELATED TO AMYLOID BURDEN COUPLED WITH IRON, ZINC, AND COPPER IN THE TRANSGENIC TG2576 MOUSE MODEL FOR ALZHEIMER'S DISEASE

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This study utilized the transgenic Tg2576 mouse model of Alzheimer's Disease (AD) that expresses the human amyloid precursor protein (APP). Iron, zinc, and copper in the brain are thought to interact with amyloid proteins, specifically Aβ, to facilitate the cognitive decline associated with AD. Transgenic (Tg) and Wildtype (Wt) control mice were given different doses of metal in their water supply beginning at approximately 3 months of age and were tested for memory impairment with a fear conditioning (FC) test at 14 months of age. The FC test was 6 minutes in duration, with the shock administered on the last 3 minutes of the Training Day (Day 1). The water groups were lab (no added metal), iron [$Fe(NO_3)_2$ at 10ppm], zinc [$Zn(CO_3)$ at 10ppm], and zinc + copper [$Zn(CO_3)$ at 10ppm and $CuCl_2$ at .25ppm respectively]. There was an unexpected difference between the genotypes on the Training Day. The Tg mice displayed abnormally low freezing

behaviors when compared to the Wt mice and it is unclear whether this was caused by plaque associated brain damage, or a differential response to the fear stimulus due to hyperactivity. Due to this difference between the genotypes an individual growth curve (IGC) analysis was conducted and indicated that there was a significant difference between genotypes, but not between water groups. In the contextual environment, the Tg mice exhibited significantly lower freezing behavior than the Wt mice. Conversely, in the cued environment the Tg mice exhibited significantly more freezing behavior than the Wt mice. This result in the cued environment was unexpected, but when the contextual freezing behavior of the Tg mice was subtracted from their cued freezing behavior, there was no significant difference between the two genotypes. This suggests that there was impaired contextual conditioning in the Tg mice and the inability to distinguish between the contextual and cued environments resulted in an additive effect on the freezing behavior of the Tg group in the cued environment. The mice were sacrificed at 18 months of age and the plaque load of the Tg lab and iron water groups was analyzed. Linear regression analyses were conducted on these water groups and a significant negative correlation was found between plaque burden and freezing behavior for minutes 456 of the Training Day (Day 1) and minutes 456 of Day 2 (the first trial in the contextual environment). Higher plaque burden was associated with a lower freezing behavior. Also the lab water group had a higher plaque burden than the iron water group in the basal ganglia and exhibited significantly less freezing on minutes 456 of Day 2 in the contextual environment. This supports the theory that a higher plaque burden is disrupting the hippocampus and possibly the basal ganglia, which results in a change in

motor behavior (hyperactivity and reduced freezing), and that the intake of metals (such as iron) could affect the deposition of plaques in specific brain areas. The overall conclusion is that when looking at the effects of water type on freezing behavior, the strong differences found between the genotypes could have overshadowed weaker differences between water types that may have been present.

1. Introduction

Alzheimer's disease (AD) currently affects millions of people worldwide. It is the most common cause of dementia in aging humans and at present there is no successful treatment (Higgins and Jacobsen, 2003; Ognibene et al., 2005). This degenerative, and ultimately fatal, neurological disease attacks the synaptic pathways in the brain and disrupts cognitive functions. As research in healthcare continues to make strides in lengthening our lifespan, AD is becoming more of a problem to the elderly population. The 65 plus age group is one of the fastest growing sections of the population in America, which means that in the near future the cases of AD will only increase. Many people are able to retain the majority of their independence in the early stages of AD, but as the disease progresses, individuals quickly lose their ability to take care of themselves.

The disease first attacks the temporal lobe, including the entorhinal cortex, hippocampus, and amygdala (Hamann, Monarch, and Goldstein, 2002). The damage continues to spread throughout the brain, causing deficits in memory, motor control, spatial awareness, and emotional stability, requiring that AD patients be monitored and cared for continuously. The immense resources that will ultimately be required to care

for our aging population could be drastically reduced if a cure or at least some treatment for AD could be found. In order to accomplish this, we must understand the mechanisms behind the pathology of AD. The two main pathological symptoms are the development of amyloid plaques and neurofibrillary tangles in the brain. The amyloid plaques contain a protein known as Aβ and the tangles contain a protein known as tau.

The Aβ hypothesis is a well supported theory seeking to explain the underlying cause of AD. It suggests that a particular protein called beta amyloid (Aβ) is responsible for the breakdown of the synaptic pathways and neuronal death through the deposition of plaques. This protein exists in different amino acid lengths ranging from 39-43 and comes from the cleavage of a normally occurring parent amyloid precursor protein (APP). If cleavage occurs by beta and gamma secretases, this produces an abnormal and harmful form of amyloid (Ohno et al., 2006). Aβ-40 and Aβ-42, referring to the length of the cleaved amino acid chain, are considered to be the major contributors to plaque formation. Alpha secretase, on the other hand, cleaves APP into a non-toxic form. Many different types of harmless amyloid are present in all biological fluids and are a normal part of a healthy brain. It is the abnormally cleaved Aβ that can cause problems, and while different lengths of amyloid protein can aggregate to form plaques in brain tissue, Aβ-42 is thought to be the most harmful (Bush, 2002). Along with these plaques, neurofibrillary tangles are also present in AD due to the abnormal hyperphosphorylation of a protein known as tau. The exact relationship between tangles and plaques and the role they both play in AD is not known, but the Aβ hypothesis presumes that the harmful

forms of amyloid appear prior to the tangles and that they are the major cause of neuronal degeneration.

Currently, there is a debate as to whether the soluble (free form) or insoluble (plaque form) Aβ-42 causes the most damage. The size and frequency of amyloid plaques in the brain (also referred to as plaque burden) does not always correlate with the observed behavioral deficits and some studies suggest that there is a stronger link between soluble Aβ and these deficits (Comery et al., 2005; Deacon, Cholerton, Talbot, Nair-Roberts, Sanderson, Romberg et al., 2008). Amyloid plaque load has not been conclusively linked to neuronal loss, number of tangles, or dementia in patients with AD (Lindner, Hogan, Krause, Machet, Bourin, Hodges et al., 2006). One study showed that in Tg mouse cortical areas, the neurons appeared to be displaced instead of destroyed by amyloid deposition. They hypothesized that small oligomers linked with the soluble to insoluble amyloid conversion caused the neural toxicity (Schwab, Hosokawa, and McGeer, 2004). Another study showed that there was no correlation between memory and insoluble amyloid deposition in Tg2576 mice when put through a Morris water maze behavioral model (Westerman, Cooper-Blacketer, Mariash, Kotilinek, Kawarabayashi, Younkin et al., 2002).

Elevated levels of soluble amyloid, on the other hand, have been related to impaired long term potentiation (LTP), a mechanism which is critical for learning and memory (Comery et al.; Quinn et al., 2006). Also, transgenic mice can show behavioral impairments before the deposition of plaques, further illustrating the possible importance of the soluble form of Aβ (Billings et al., 2005; Comery et al.; Selkoe, 2002). The

Tg2576 mouse has shown cognitive deficits in multiple behavioral tasks that were related to the level of soluble amyloid and not to the plaque burden even though the levels of soluble amyloid were comparatively small. This could indicate a more important role for soluble amyloid in the pathology of AD (Lindner et al., 2006). It is difficult, however, to decipher which form poses the biggest threat due to the concurrent existence of multiple forms in the brain at any given time including monomers, oligomers, and fibrils (Selkoe, 2002). Another area of contention is whether intracellular or extracellular Aβ causes the most damage and by what mechanism (Billings et al.; Golde and Janus, 2005; Smith, Green, and LaFerla, 2005). Regardless of this debate, there is an ever increasing body of research that continues to support the idea that there is a relationship between amyloid and AD (Hardy and Selkoe, 2002).

Transgenic Mouse Models

Transgenic (Tg) mice are currently one of the main avenues of research focusing on understanding the pathology of AD. Through the expression of what is thought to be the key genetic factors underlying AD, genetically modified animal models can provide valuable information on the mechanisms of the disease and the possible success of new treatments (Spires and Hyman, 2005; Higgins and Jacobsen, 2003; Wu et al., 2006). The mouse involved in this study is the Tg2576 transgenic mouse expressing the human APP gene with the Swedish mutation. The Swedish mutation is a two-point mutation involving a replacement of lysine with asparagine and methionine with leucine (Ognibene et al., 2005). This mouse model has been shown to mirror some of the

pathological symptoms of AD due to amyloid build up and the development of amyloid plaques in the brain (Hsiao et al., 1996). Elevated Aβ levels can be found as early as six months and by ten months neuritic plaques can be seen in the hippocampus and neocortex (Ognibene et al.).

Neuritic plaques, also known as senile plaques and dense core plaques, are extracellular deposits of amyloid found in gray matter that contain dead neurons and have been associated with neuronal death and AD. Congo red staining is a common technique used to identify amyloid plaques in brain tissue. Amyloid proteins congregate to form fibrils and then these fibrils gather together to form a complex structure called a beta pleated sheet. The beta pleated sheet consists of multiple folds of fibrils and it is these folds that attract and bind to the congo red stain. After staining, amyloid deposits in senile plaques and in the walls of blood vessels become bright red in color. Also, if viewed under polarized light, the stained amyloid is seen as bright green due to the birefringent nature of senile plaques. In this way, two techniques can be used to identify senile, dense core plaques and distinguish them from more diffuse deposits of amyloid and artifacts that may be present in the tissue.

The deficits in animal models, however, are not an identical representation of those associated with AD in humans, but many adequately approximate enough of the pathology to make them helpful research tools (Janus and Westaway, 2001; Quinn et al., 2006). For example, neurofibrillary tangles, an inflammatory response, and neurodegeneration, hallmarks for AD in humans, are not usually present in Tg mouse models and are not seen in the Tg2576 mouse model (Higgins and Jacobsen, 2003;

Maynard et al., 2002; Schwab et al., 2004). Also, some studies have found no deficit in the Tg2576 mouse model at all for certain behavioral tasks. Bizon, Prescott, and Nicolle (2007) found no impairment in the 15 month old Tg group compared to a control group testing hippocampal dependent behavior in T-maze and water maze models. According to their study, this may be due to a retention of brain function despite a large amyloid burden, or the use of non-spatial or non-hippocampal learning strategies. King and Arendash (2002) did not find any widespread cognitive impairment in 19 month old Tg2576 mice for a variety of behavioral tasks including Y-maze, visible platform, Morris water maze, circular platform, passive avoidance, and active avoidance. Another study suggests that prolonged exercise can rescue some of the cognitive deficits in the Tg2576 mouse. Nichol, Parachikova, and Cotman (2007) found that only three weeks of wheel running caused the Tg mouse group to become indistinguishable from the Wt group on radial arm and water maze tasks. This showed that exercise could be beneficial even after the onset of severe pathology and perhaps regular exercise is one reason that behavioral deficits are sometimes not seen.

That is not to say that behavioral deficits are never seen in the Tg2576 mouse. It is not a perfect representation of AD in humans, but it does successfully approximate some of the pathology. One behavioral study conducted by Ognibene et al. (2005) that utilized mazes showed a deficit in both working and spatial memory in Tg2576 mice. They also found that the Tg2576 mice were more active and displayed a disrupted circadian rhythm when compared to controls. This reflects the same altered activity pattern that is seen in many AD patients. Other deficits may be present due to the genetic

background of these mice, including blindness and disinhibition. Motor impairment has also been documented in some APP transgenic mouse models (Wirths and Bayer, 2008).

Hyperactivity, or simply increased or higher than normal activity, has been seen in the Tg2576 mouse in open field tests (Gewirtz et al., 2000; Gil-Bea, Aisa, Schliebs, and Ramirez, 2007; Lalonde, Lewis, Strazielle, Kim, and Fukuchi, 2003), home cage behavior, novel object recognition tests (Ognibene, Middei, Daniele, Adriani, Ghirardi, Caprioli et al., 2005), elevated plus maze (Gil-Bea et al., 2007), Y-maze (King and Arendash, 2002), and motor impulsivity tests (Adriani, Ognibene, Heuland, Ghirardi, Caprioli, and Laviola, 2006). This increase in activity is thought to be related to disinhibition, a characteristic common in humans suffering from AD, characterized by an increase in behaviors that would be inappropriate for a normal individual. One example for mice would be an increase in the frequency of choosing open arms in a maze when mice normally prefer closed in spaces (Ognibene et al.). Many people suffering from AD will wander and if not closely watched they could get lost or more importantly injured by walking into a busy street or a construction zone. A loss of inhibitory control has also been attributed to the frequent outbursts of agitation in the AD population, as well as other socially unacceptable behaviors and inappropriate euphoria (Adriani et al.; Lalonde et al.).

Amyloid is present in non-transgenic rodents, but they don't develop the plaques that have come to be associated with AD in humans without the addition of the human genes. Normal rat and mouse Aβ contains certain amino acid substitutions that make these forms of amyloid harmless even in the presence of metals. The interactive

properties that are most responsive to metals are not present in Wt rat and mouse amyloid and this may explain why they are immune to the negative age-related effects seen in humans and other mammals. It is only when human genes are introduced into Tg mice that we see some of the deficits corresponding to age-related AD in humans (Bush, 2002; Inestrosa et al., 2005).

The amyloid peptides that are expressed in this Tg mouse model are physically and chemically different from those found in humans with AD. Humans have dense core plaques that are insoluble in certain detergents, however the amyloid plaques in the mouse model are soluble in these solutions, illustrating the differences in these structures. Also, the mice exhibit a higher level of soluble amyloid with a lower level of vascular deposition when compared to humans with AD (Kalback, Watson, Kokjohn, Kuo, Weiss, Luehrs et al., 2002). Such differences in the structure and deposition of amyloid must be taken into consideration when evaluating results based on this mouse model.

The use of Tg mouse models such as Tg2576 only tests the amyloid hypothesis of AD. If there are other factors unique to human physiology involved, they would not be shown in this model. One alternative is to use a mammal that naturally exhibits the symptoms of AD as it ages. This may be possible with the discovery of Octodon degu, a South American rodent that exhibits both amyloid plaques and tau protein aggregates as it ages (Inestrosa, Reyes, Chacon, Cerpa, Villalon, Montiel et al., 2005). Tg animals offer a precise way to study the effects of specific genes, but naturally occurring models may provide a more accurate picture of the mechanisms behind AD. While this study

used Tg mice to focus on the specific nature of APP and metals in the brain, the use of many other models of the disease is necessary if we are to fully understand AD.

Metals and AD

Among the factors believed to contribute to AD, the presence of different metals in the brain is also a major focus of current research. The levels of iron, zinc, and copper are thought to have severe impacts on the progression and pathology of AD. All three are found in high concentrations within, and in close proximity to, amyloid plaques. It is thought that an interaction with these metals facilitates the aggregation of Aβ (House, Collingwood, Khan, Korchazkina, Berthon, and Exley, 2004; Huang, Moir, Tanzi, Bush, and Rogers, 2004; Maynard et al., 2002; Suh et al., 2000). In fact, it has been found that in the absence of these metals, the Aβ protein is monomeric and does not aggregate into harmful oligomeric forms (Bush, 2002; Ong and Farooqui, 2005). These three metals are all essential to the normal functions of the body, but at certain levels they may exacerbate the cognitive decline associated with AD. This is especially important for our elderly population that is usually encouraged to take multiple supplements daily that may include these metals. In an effort to supply them with the necessary nutrition, we may be aiding in their mental decline. The levels of iron and copper in the brain can increase naturally as a function of aging and one hypothesis suggests that the formation of amyloid plaques is a direct result of an interaction between the amyloid protein and excessive levels of these metals in the brain. Iron has been specifically related to AD in that twice as much was found in the gray matter of demented AD patients when compared to non-demented

controls (Falangola, Lee, Nixon, Duff and Helpern, 2005). This age dependent amyloid/metal interaction is believed to cause oxidative damage, resulting in the pathology of AD (Bush, 2002; Bush, 2003).

Copper and iron are both redox active and are important players in many enzymatic reactions in the body (Bush, 2000; Bush, 2003; Maynard et al., 2002; Ong and Farooqui, 2005). Without proper regulation of the levels of these metals, oxidative damage can occur and this specific type of damage has been found in AD brains (Maynard et al., Ong and Farooqui). Aβ has been shown to interact with metals in the brain, aiding in its aggregation and causing the release of toxic levels of hydrogen peroxide (Bush, 2002). Tg2576 mice over expressing APP have shown reduced levels of free copper and zinc, probably due to the presence of copper/zinc binding sites in the Aβ region. Humans with AD show a significant increase in cortical iron levels with deposits found in neurofibrillary tangles, glial cells, and amyloid plaques (Ong and Farooqui). For the mouse model, iron levels remained fairly constant in all but the oldest mice (18+ months), where a marked increase was observed. Treatment of these mice with a metal chelator showed a significant decrease in amyloid deposition. This can be difficult to interpret, however, because it's not always clear which metals are being chelated and to what degree. It may be beneficial to focus on ways to decrease the number of plaques and increase the cellular maintenance of proper levels of metal in the brain (Maynard et al.). Other mouse models of AD have also shown co-localization of iron with the amyloid plaques (Falangola et al., 2005).

The natural increase in metals in the brain could be the driving force behind the neuropathology associated with AD. The hydrogen peroxide released by the interaction of copper and iron with amyloid can interfere with the body's ability to clear amyloid from the brain tissue. Zinc is also thought to be important through its interaction with copper/iron modified amyloid, facilitating its deposition into plaques. The locations containing the highest concentration of zinc in the brain also correspond to the locations of greatest damage in AD (Bush and Tanzi, 2002). The effects of zinc have been seen in the Tg2576 model. For example, age is more directly related to an increase in zinc concentrations in the brains of female Tg2576 mice and consequently the Aβ burden is higher in these mice (Bush, 2003; Maynard et al., 2002). This may reflect the fact that AD is more prevalent in women than men and could be the result of a more active zinc transporter in women (Bush).

The role of copper in AD pathology is not completely understood. We do know that extracellular copper can interact with Aβ to aid in the formation of harmful oligomers. This leads to a decrease in copper levels that has been reproduced in several different strains of transgenic mice (Maynard, Bush, Masters, Cappai, and Li, 2005). When you take these copper deficient transgenic mice, however, and supplement them with copper, there is an improved survival rate accompanied by a significant decrease in both soluble and insoluble Aβ. This particular effect is only present in APP transgenic mice and suggests that perhaps copper can drive an alternative APP pathway that does not lead to the production of harmful forms of Aβ. It has been suggested that the amyloid pathway directly involves the natural copper regulating machinery in the brain and that

the disruption causes a copper deficiency which in turn promotes the continuation of the amyloid pathway (Maynard et al.). Therefore, even though extracellular copper can interact with amyloid to produce negative effects, an increase in intracellular copper may serve to downregulate the production and deposition of amyloid.

The oxidative damage due to the amyloid/metal interaction affects many parts of the brain, including the hippocampus and the amygdala. These areas are associated with learning and memory and the amygdala in particular is involved with emotional arousal and memories related to high anxiety or fear. It has been shown that humans suffering from AD, with decreased amygdalar volume, have impaired emotional memory involving traumatic events (Mori, Ikeda, Hirono, Kitagaki, Imamura and Shimomura, 1999). Individuals with AD who experienced the 1995 earthquake in Japan were interviewed to assess their memory of the incident and then magnetic resonance imaging (MRI) was used to measure the volume of both the hippocampus and the amygdala. Emotional memory was found to be correlated with amydalar volume (Mori et al.). In normal individuals, memories involving emotional situations are enhanced and often easier to retrieve (Hamann, Monarch, and Goldstein, 2000). Fear conditioning is one way to assess learning and memory surrounding an emotional event.

Fear Conditioning

Fear conditioning is a classical conditioning behavioral test usually involving an auditory stimulus and a mild to moderate foot shock for animal models. With delay fear conditioning, the goal is to pair a tone (cue) with a shock with no intervening interval.

This is easily done by ensuring the tone and shock overlap in time. This is done in a specific environment (context) and then the level of fear elicited by the context and the cue are tested separately. All conditioning discussed will be delayed fear conditioning unless otherwise stated. A normal rodent response to a fearful stimulus, in this case a shock, is to freeze. Freezing is described as no visible movement other than respiration (Dong et al., 2005). Using video equipment and specially designed software, we can monitor a mouse's behavior during a fear conditioning experiment and quantify the amount of freezing behavior present.

Acquisition is the phase in which the mouse learns to associate the auditory stimulus with the foot shock. A normal healthy mouse will be conditioned after just a few pairings of the tone and shock. If conditioning is successful, a fear response will be elicited by the tone alone and no shock is needed. After repeated tones that are not paired with a shock, however, the conditioning will begin to extinguish. This is the phase where the mouse learns that the tone is no longer paired with the shock and the tone alone will eventually no longer cause a fear response. By comparing the length of the acquisition and extinction phases, the level of cognitive functioning of the mouse groups can be determined.

The conditioning of fear to the context of the environment, or to an auditory cue, is associated with the amygdala. A lack of freezing to either of these stimuli could indicate damage to the amygdala. However, a lack of freezing to the context alone is thought to be associated with damage to the hippocampus (Ammasari-Teule, Restivo, Pietteur, and Passino, 2001; Barnes and Good, 2005; Phillips and LeDoux, 1992).

Experiments involving lesions to the hippocampus in mice and rats have shown that in fear conditioning they will forget the location that they received the shock, but will still react to the cue that was paired with the shock (Gerlai, 2001; Phillips and LeDoux, 1992). This is explained through the existence of different forms of memory and the functions of different areas of the brain. Declarative memory deals with facts and events and is dependent upon the hippocampus. More specifically, it is the episodic portion of declarative memory (remembering specific events) that relies almost completely on the hippocampus (Vargha-Khadem et al., 1997). Contextual fear is therefore hippocampus dependent because it relies on the animal's memory of specific events and spatial awareness. The hippocampus contributes to fear conditioning through relaying sensory inputs to the amygdala and so both the hippocampus and amygdala are necessary for contextual fear (Phillips and LeDoux). Non-declarative memory involves skill learning, emotional learning and conditioning. These behaviors are supported by various other brain regions such as the amygdala and basal ganglia and are therefore unaffected by hippocampal damage (Clark and Squire, 1998).

Animal models have been found to mimic fear conditioning in humans (Hamann, Monarch, and Goldstein, 2002). The underlying mechanisms that fuel conditioned fear responses remain fairly constant across different species. Neuroimaging studies conducted on humans have supported this idea by showing that the same brain areas are involved in fear conditioning with humans as with lab animals. Of all the brain regions, the amygdala is the most important involved in fear conditioning. Any lesions to this particular area can cause deficits in conditioning and it has been shown that during the

early stages of AD, one of the areas that suffer is the amygdala (Hamann et al.). In fact, AD patients tested with fear conditioning were found to be deficient when compared to normal individuals in the same age group. All of the control subjects exhibited the conditioned response, compared to only 40% of the AD patients (Hamann et al.). All the AD patients involved in this study were in the early stages of AD, suggesting that fear conditioning may be a good measure for early detection.

The acquisition and extinction of fear conditioning in mice can be analyzed in order to assess deficits in various regions of the brain, including the hippocampus and amygdala, which as mentioned before are involved in learning, memory, and fear. These areas are greatly affected in humans during the progression of AD. With this behavioral test, we can examine some of the cognitive deficits in mice raised under different conditions. These deficits are thought to be somewhat analogous to those associated with AD and can be used to gain a better understanding of the nature of the disease.

It has been shown through previous research that the Tg2576 mouse can display significant deficits in both contextual and cued fear conditioning when compared to wild type controls (Dong et al., 2005). Different factors can influence the behavioral deficits displayed. First, the age of the mouse can significantly affect its performance. Younger transgenic mice around 3-4 months old have been found to show both contextual and cued conditioning, whereas 16 month old mice were found to only exhibit contextual conditioning (Barnes and Good, 2005). This impairment in cued conditioning probably represents damage to the amygdala, not the hippocampus. One suggested explanation has been the overshadowing of the cued auditory stimulus by the contextual stimulus. This

theory states that if the level of freezing elicited by the context is significantly higher than that of the cued stimulus, this can cause the context to overpower the cue and a differential response is seen. This would make the conditioning of one stimulus dependent on the strength of the association with the other. Previous experiments have found impairment in contextual, but not cued conditioning in these mice (Barnes and Good; Quinn et al., 2006).

Another slightly different explanation is that one particular stimulus in the context (and not the context itself) was associated with the shock and that this stimulus was not present in the cued environment. A mouse with a damaged hippocampus may still freeze during a contextual fear conditioning experiment if the context contains a salient cue that could be associated with the shock. For prey animals, an example could be any dark shapes visible that resemble predators, such as two dark spots representing eyes. That cue could be paired with the shock in the same way as the tone. Consequently, that mouse may exhibit freezing behavior in the contextual condition, not because it remembers being in that environment, but because it remembers that specific cue. This is dangerous because while the experimenter thinks contextual conditioning is being measured, the mouse is really responding to a cue. A highly salient cue should be paired with the shock and all other possible cues eliminated from the contextual environment (Gerlai, 2001). It would seem that the detrimental effects of the mutation are not static, but can be dependent on the saliency of the stimuli presented.

Another factor that can influence behavioral deficits is the number of pairings between the conditioned (CS) and unconditioned stimulus (US) during the training phase.

Tg2576 mice can show deficits in contextual fear conditioning by five months of age, but it can be overcome by increasing the number of CS-US pairings from two to seven during training. With the increased pairings, these mice were not significantly different from the controls (Comery et al., 2005). This difference only occurs in younger mice, because the same experiment performed on nine month old Tg2576 mice showed a constant impairment in contextual conditioning with or without an increase in the number of CS-US pairings. No significant difference was found for cued conditioning (Comery et al.).

The time interval between the training phase and the first testing phase can also affect the results of behavioral testing. With a span of less than 20 hours between the training and testing, five month old Tg2576 mice were comparable to the controls for contextual fear conditioning. With a span of more than 20 hours, however, they showed a significant impairment (Comery et al., 2005). Many factors can influence the observed behavior of Tg mice and they must be taken into consideration when designing any study that focuses on impairments in conditioning.

All the previous studies have dealt with delay conditioning, where the tone and shock are paired together at the same point in time. Trace conditioning involves a gap between when the tone is heard and when the shock is delivered. This time gap requires hippocampus dependent temporal memory processing in order for an animal to exhibit freezing behavior (Clark and Squire, 1998). Trace conditioning was applied to 4-6 month old Tg2576 mice and it was found that they were unable to associate the tone with the shock due to the time difference (Ohno et al. 2006). This suggests that the Tg2576 mouse suffers hippocampal damage much earlier than traditional delay conditioning

would show and also much earlier than the detection of plaque formation. For delay conditioning, the Tg2576 mice performed comparably to the wild type controls (Ohno et al.).

Delay fear conditioning can be used to study both hippocampal and amygdalar damage in Tg2576 mice given that they are of sufficient age to exhibit the deficits associated with brain damage. This type of conditioning is especially sensitive to amygdala damage since it has been shown that the amygdala is responsible for encoding memories associated with fear stimuli (Fanselow and Gale, 2003). Memory impairment is a major symptom of AD and using fear conditioning to study the effects of damage to the hippocampus and amygdala in mice may give us some insight into the emotional memory systems of humans with AD. This experiment specifically focuses on different types of metals in the brain and how they may interact with the amyloid plaques to cause differing behavioral responses to fear stimuli in the Tg2576 mouse model for AD.

People suffering from AD have many different cognitive deficits, including emotional learning and memory. We know a lot about the cognitive decline associated with AD, but we do not currently know the relationship between amyloid plaques and levels of zinc, iron, and copper in the brain. We do not know if the levels of certain metals in the brain cause a change in plaque pathology and memory loss. If so, we may need to limit the consumption of, or exposure to, certain metals in those suspected at risk of developing AD. We hoped to learn more about the degenerative progress of this disease by uncovering the antagonistic effects of metal.

2. Experiment

In an attempt to gather more information, delayed fear conditioning techniques were used on Tg2576 mice expressing human APP and Wt2576 normal wildtype (Wt) mice. Behavioral data was compared with histological data concerning the amount of amyloid plaques found in different areas of the brain. It was expected that the Wt mice on any metal will show lower freezing behaviors due to higher memory loss when compared to the Wt mice on plain water. This difference was expected to be even more pronounced when comparing the transgenic mice on metal to those on plain water. The metals may interact with the amyloid causing accelerated plaque formation. The transgenic mice on metal were thus expected to have a higher plaque burden in the hippocampus and amygdala. Copper was expected to have a beneficial effect on memory and plaque burden.

This study includes both Tg and Wt mice that were raised on regular water, or water containing different metals. The groups with metal added to their water include iron, zinc, and zinc + copper. Once the Tg mice were old enough to begin exhibiting the signs associated with cognitive impairment (approximately 14 months), all the mice were tested in a fear conditioning chamber. The level and speed of conditioning achieved by

the different groups were analyzed to see if there was any significant difference between them. This information could help us understand the behavioral and neurological changes associated with increased amyloid burden in the brain, increased levels of metal in the brain, and a combination of the two. Once the behavioral testing was complete, the mice were sacrificed (at approximately 18 months of age), and a histological analysis was performed. Congo red staining techniques were used to look for amyloid in the brain. Dense core plaques are easily visualized due to their birefringent nature. By using a polarized scope, dense core plaques will show up as bright green.

The goal of this study was to gather fear conditioning and histological data indicating the specific interaction effects of amyloid and metals such as iron, zinc, and copper using the Tg2576 transgenic mouse model for AD. Many pharmaceutical treatments focus on the metal binding sites of Aβ and if we could discover which metals are related to behavioral deficits and increased brain damage, this can be the premise for treatments designed to combat those specific metal interactions (Bush, 2002).

Hypotheses

It was expected that the wild type mice on plain lab water would be the fastest to both acquire and extinguish conditioning. The Tg mice were expected to show poor conditioning due to damage to the hippocampus and amygdala from amyloid deposition. The most impaired mice were expected to be the Tg's on zinc, since elevated levels of this metal has been shown to cause cognitive deficits (Maynard et al., 2005). In the Tg mice, this may be due to its combination with human APP to promote the aggregation of

amyloid into plaques. It is also hypothesized that the copper in the "zinc + copper" water would rescue some of the deficits and so Tg mice on this water would outperform the Tg mice on zinc alone. The effects of iron were unknown and no hypothesis was made concerning those groups. The mice performing the worst on contextual and cued fear conditioning were expected to have a heavier plaque burden.

Method

Subjects

The current study involved a cohort of 83 female transgenic (Tg2576) and wild type mice obtained from Taconic at approximately three months of age. It was split further into two groups labeled A and B. These groups were counterbalanced for water type and genotype. The separation was necessary due to time constraints with the procedure. There weren't enough hours in the day to put all 83 animals through the apparatus, but by splitting them into two groups they could be run in back to back trials for 12 days (6 days for Group A and 6 days for Group B). They were raised on plain water, iron water, zinc water, or zinc plus copper water. The 10ppm $Fe(NO_3)_2$ iron mixture was made by adding 10mL of $Fe(NO_3)_2$ to 10L of water. The zinc water was 10ppm $Zn(CO_3)$, which was made by adding 10mL of $Zn(CO_3)$ to 10L of water. The zinc plus copper water was made by adding 10mL of $Zn(CO_3)$ and 250µL of $CuCl_2$ to 10L of water. Table 1A lists the division of animal groups that were involved in both experimental runs (Groups A and B) during the study. One mouse died, 10 were

removed from the study due to a genetic predisposition for blindness, and 1 mouse had to be reassigned due to incorrect genotyping, leaving 72 mice. Table 1B lists the combined census of Groups A and B with the removal of blind mice and the reassignment of one mouse (Group C).

Table 1A. Animal Groups For Both Experimental Runs

Group A

	Zinc	Zn+Cu	Iron	Plain
Transgenic	5	5	6	6
Wildtype	6	5	5	4

Group B

	Zinc	Zn+Cu	Iron	Plain
Transgenic	5	4	5	4
Wildtype	5	6	5	7

Table 1B. Animal Groups For Statistical Analysis

Group C

	Zinc	Zn+Cu	Iron	Plain
Transgenic	7	9	10	10
Wildtype	9	11	8	8

Apparatus

The mice were housed in a 12 hour light/dark cycle and run in dedicated space in David King Hall on the George Mason University Campus in Fairfax, Virginia. The fear conditioning room consisted of two soundproofed boxes (provided by Coulbourn Instruments), each containing a cage hooked up to a computer that is capable of producing a soft or loud tone, low or high illumination, and a foot shock of between 0-3

milliamperes in intensity. Each box also had a video camera connected to the computer to record videos of the mice in mpeg format. The video was run through specialized software created by Clever Sys Incorporated designed to measure freezing behavior. Figures 1 and 2 show the fear conditioning boxes and the computer used to run the experiment. Plexiglas floor covers were used to cover the bars of the cage during the cued phases of the experiment. Black laminated shapes, mouse bedding, and a white noise generator were also used.

Figure 1. Fear conditioning boxes and computer system

Figure 2. Fear conditioning boxes and cages

Procedure

The mice were tested for cued and contextual delay fear conditioning (see Figure 3). Pilot work was completed using white mice to select an appropriate shock level and duration. Each grouping of mice was run separately and each series of trials took approximately one week. On Day 0, the mice were habituated to both the contextual and cued environments. After being brought back to a holding room and allowed ten minutes to habituate to the change, the mice had six minutes in each environment. Every trial during the experiment was six minutes long with a ten second buffer time at the beginning to allow time for the experimenters to close the doors to the boxes and leave the room if they wished. The contextual environment consisted of a bare cage on the left hand side of the sound proof box with exposed bars as the floor, no white noise, high light inside the box, and high light in the FC room itself. The cued environment consisted of a Plexiglas floored cage on the right hand side of the sound proof box with soft bedding placed on top of the Plexiglas, white noise running in the background, high light inside the box, low light in the FC room itself, and black laminated shapes taped to three of the walls of the cage. Figure 3 illustrates the two different environments.

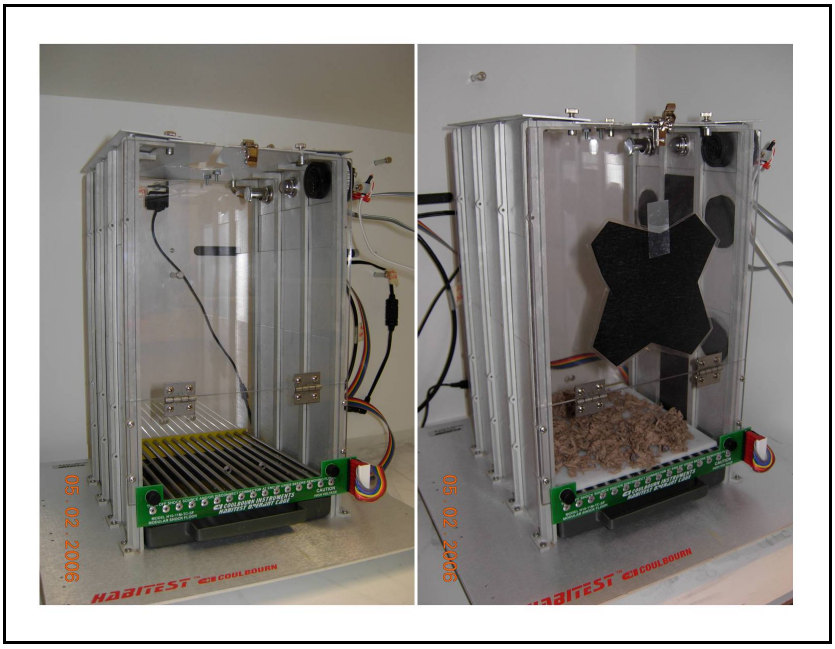


Figure 3. Contextual and Cued Environments

On Day 1, the mice were classically conditioned through the pairing of a tone with a shock in the contextual environment on the left in Figure 3. The computer was set to deliver a 20 second 85 decibel tone at 170, 230, and 290 seconds. During the last 2 seconds of each tone, a .75mA shock was delivered to the mice's feet through the metal grid comprising the floor.

On each consecutive day thereafter, the mice were tested for contextual and cued conditioning. The order in which the mice were run each day was changed to control for the time of day each mouse went through its trials and the different groups were counterbalanced. Every mouse spent six minutes in both environments during each day. The contextual environment was tested first in the morning, followed by the cued environment in the afternoon. For the contextual environment the mouse was placed in the same setting where it was given the shock, but no tone or shock was presented. For

the cued environment, the tone was presented at 170, 230, and 290 seconds, but without the shock. Day 2 was testing the acquisition of fear to the two environments. Since the mice were not shocked any further, every day afterward was considered an extinction trial. The conditions on Day 2 were repeated on Days 3, 4, and 5. See Table 2 for an outline of the procedure. Once all behavioral work was complete, the mice were euthanized and their brains extracted, frozen in dry ice, and placed in a -80°C freezer. They were sliced and mounted on slides followed by congo red staining focusing on amyloid plaques. Figure 4 shows an example of congo red staining with the bright red spots being amyloid plaques.

Table 2. Fear Conditioning Procedure

Day	Trial Type	Time
0	Habituation	6 min in each environment
1	Training	6 min conditioning
2	Contextual/Cued: Testing	6 min in each environment
3	Contextual/Cued: Extinction	6 min in each environment
4	Contextual/Cued: Extinction	6 min in each environment
5	Contextual/Cued: Extinction	6 min in each environment

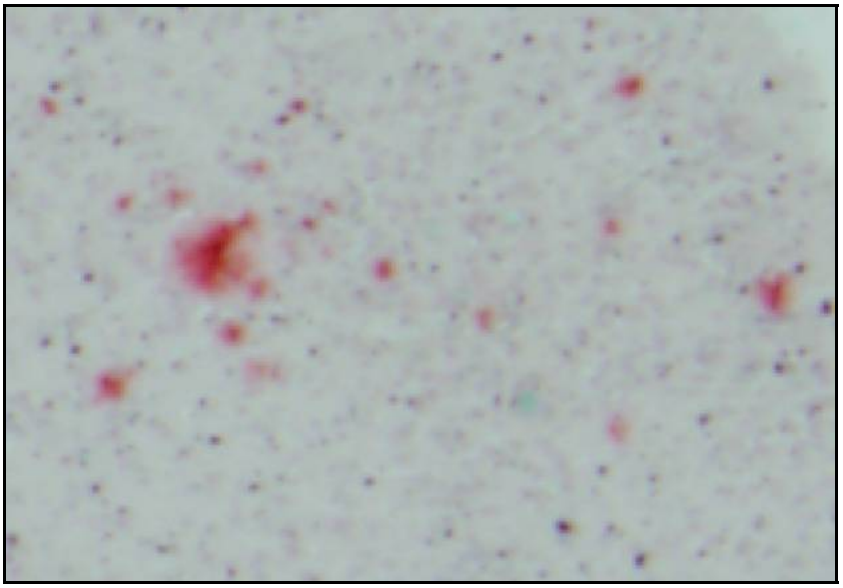


Figure 4. Magnified tissue with visible amyloid plaques stained by congo red

The Freezescan computer program then analyzed the video taken during the trials. The freezing behavior was measured in 60 second intervals for the full six minutes and the percentage of time spent freezing was computed. The criterion for the beginning of a freeze event was less than 40 pixels of illumination change in 22 out of 24 frames. The criterion for the end of a freeze event was more than 40 pixels of illumination change in 8 out of 10 frames. The video was taken in 300 frames per second. Figure 5 shows the main window of the Freezescan program as it looked prior to the start of the experiment.

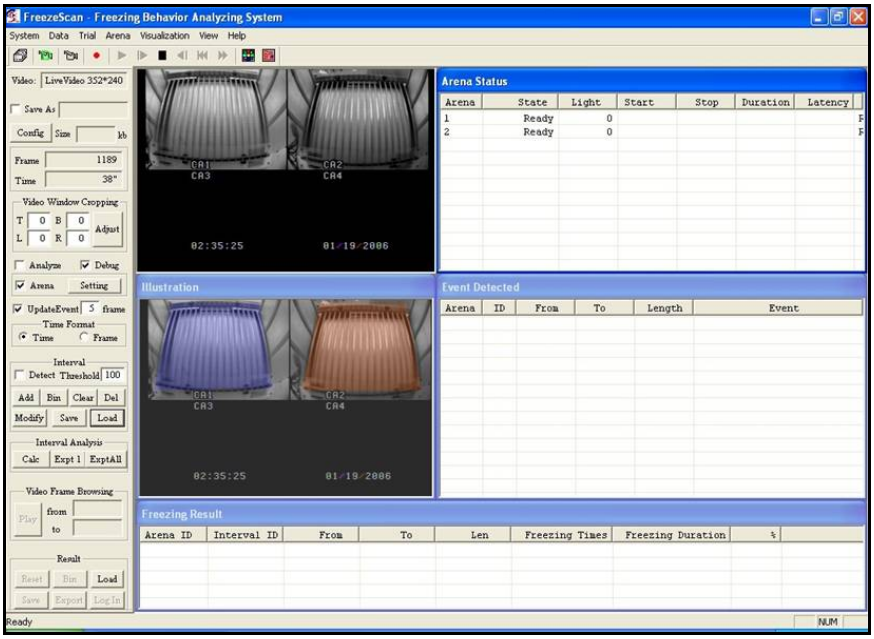


Figure 5. Main window of the Freezescan program used to analyze video data

Data Analysis

Repeated Measures ANOVA's

The data from Freezescan was exported to SPSS and multiple analyses were performed. A repeated measures analysis was first conducted to compare the DV (mean percent freezing: the percentage of time spent in a motionless state) of the various groups (IV's genotype and water type) across both days (1-5) and minutes (1-6) within days. The "genotype" refers to Tg versus Wt animals. The "environment" refers to the contextual and cued environments. The data was separated in this way because memory relating to the environmental context and memory relating to a learned cue utilize different parts of the brain. The "water type" refers to lab, iron, zinc, and zinc + copper

water groups. This was done first in order to assess any obvious differences between the groups. The six minute trials were split into the average of the first three minutes and the average of the last three minutes. There were six minute trials for both environments (contextual and cued). The shock stimulus always took place in the contextual environment during the last three minutes of the trials on the training day. Separating the trials this way allowed us to see if the animals were anticipating the shock during the entire trial or only during the latter half of the trial. The two groups (A and B) that were created due to a time constraint in the experimental procedure were merged into a single group for analyzing purposes since no significant differences were found ($F_{(1, 64)} = .438$, $p = .510$ for contextual and $F_{(1, 64)} = .231$, $p = .632$ for cued). Both the color of the mouse ($F_{(1, 64)} = .156$, $p = .694$ for contextual and $F_{(1, 64)} = 1.273$, $p = .263$ for cued) and the particular box it was placed in ($F_{(1, 64)} = 1.115$, $p = .295$ for contextual and $F_{(1, 64)} = .106$, $p = .746$ for cued) were disregarded since no significant differences were found. A repeated measures ANOVA was used to test for significant differences between the freezing behavior of these groups with the average of the first and last three minute periods for all trial days and a significance level of $p < .10$ due to the low sample size.

Individual Growth Curves

An individual growth curve (IGC) analysis was chosen over a more traditional fixed effects analysis (such as ANOVA) to compare freezing behavior of the genotype and water type groups for several reasons. The differences between the water groups were expected to be small and an initial ANOVA showed that the genotypes were

significantly different on the training day. There was a possibility that this difference could overshadow the water group differences and so the use of individual growth curves was needed to ensure the maximum capacity to detect any significant differences. A repeated measures analysis of covariance (ANCOVA) was the traditional method initially considered for analyzing the data gathered, but the underlying assumptions regarding change over time were not compatible with the current study.

One of the assumptions of the ANCOVA is that the change over time for individuals is roughly equal and while their starting point may differ, the increase or decrease in the measured behavior will be similar across all members of a group (Girden, 1992; Willet, Singer, and Martin, 1998). For example, one mouse may change from freezing 10% to 20% over one day of trials while another mouse may change from 60% to 70%. Even though their starting rates were very different (10 and 60), their freezing behaviors increased by the same amount (10). The assumption would be that all the mice would increase by roughly 10 percentage points over the course of one day of trials. Through observation of the mouse groups involved in this study, it became quite clear that their individual behaviors not only were significantly different at the start of the experiment (on the Training Day), but that the way in which their behavior changed over time was also different. Therefore, if we were to use the traditional method, this assumption would be violated and the results may have been difficult to interpret or even misleading.

Traditional methods often rely on means including mean rates of change over time (average slopes). Any variance about the means is incorporated into the error term,

and consequently those differences are not taken into consideration in the analysis (Rogosa, Brandt, and Zimowski, 1982). Since individual differences are of great importance in this particular type of study, it makes more sense to use a method that tests these individual differences instead of ignoring them. If some individuals in a group change their behavior in a particular direction and others change in the opposite direction, the average slope depicted in a traditional analysis would be misleading. By using IGC, all the data points for each individual gathered over the length of the study can be incorporated to describe the individual's change in behavior over time (Lawrence and Hancock, 1998). They become the parameters of interest and include the intercept and slope, which represent the starting point and the rate of change over time, respectively.

The IGC analysis is conducted in two steps, involving two models referred to as Level 1 and Level 2. The Level 1 model, also known as the within-subjects model, is where individual growth parameters are obtained for each mouse through the use of individual regressions in which time predicts the DV. Taking the data into account, a specific functional form is assumed to incorporate the majority of the individual growth curves (Willett et al., 1998). In this case, the assumption was that a 2nd order polynomial best fitted the data ($DV = b_0x^0 + b_1x^1 + b_2x^2 + \varepsilon$). The dependent variable was percent freezing which is regressed on the intercept (baseline) plus the linear and quadradic predictors and any residual error. This was assuming that there was an initial steady increase in the rate of freezing behavior after the delivery of a shock (linear), and a change in the rate of freezing behavior as it decreased over time without shocks being given (quadratic). So, the growth curve analysis was made up of the intercept, linear, and

quadratic components, which characterize change in the Dependent Variable (percent freezing). With each individual having their own growth curve, any variance between these curves reflects a difference in how those individuals changed over time.

For the Level 1 analysis, the data was separated into minutes 123 and minutes 456, both environments (contextual and cued), and the linear and quadratic components. These components were coded separately for each of the three minute periods (minutes 123 and 456). Percent freezing was the DV and the IV's were genotype, water type, and time. Genotype and water type were the between subject factors, while time was the within subject factor. Individual regressions were run and new data sets were created for each of the two environments and two time periods.

In the Level 2 model, also known as the between-subjects model, the individual growth curve parameters produced from the Level 1 models were used as the Dependent Variables (instead of percent freezing) and also as covariates in three separate general linear models (GLM's). The first GLM involved the intercept, the second involved the linear component with the intercept as a covariate, and the third involved the quadratic component with both the intercept and linear components as covariates. The Level 2 model predicted these growth parameters for each mouse using the previous growth curve parameters along with genotype, water type, and their interaction as predictors. By using all of the data points for each individual in an IGC analysis, instead of simply the means of each group, the results should be more reliable than that of a more traditional method (Rogosa et al., 1982). Additionally, by choosing an IGC analysis, we were able to model individual differences in change, which are the focus of this study.

For the Level 2 analysis, the first GLM was conducted using Type III sums of squares (when we add covariates, we switch to Type I), the intercept from the Level 1 analysis as the DV and both water and genotype as fixed factors. This was then followed by a GLM with the linear component as the DV, the intercept as a covariate, water and genotype as fixed factors, and using Type I sums of squares (to hierarchically partition variance and avoid multicollinearity). A third GLM was conducted for the quadratic component as the DV, the intercept and the linear components as covariates, and water and genotype as fixed factors. Planned comparisons were conducted to compare water groups. It is important to note that we were focusing on Days 2 through 4 (the extinction days) with the IGC and the Training Day (Day 1) was not included. Both mouse groups behaved differently on the Training Day (less freezing) than they did on the following days (which is to be expected in fear conditioning) and by not including this first day, the analysis becomes more straightforward and easier to interpret.

Plaque Load and Behavior Comparisons

For the analysis of plaque load (quantified by the analyzing program imageJ), GLM's and linear regressions were used to compare the iron and lab water groups with their fear conditioning behavior (contextual and cued freezing across days). The iron group was chosen first because it is thought that iron plays a role in the formation and stabilization of beta pleated sheets (House, Collingwood, Khan, Korchazkina, Berthon, and Exley, 2004). Perhaps the iron actually facilitates the condensation of amyloid into

beta pleated sheets leading to the development of dense core plaques. Analysis of the plaques in the zinc and zinc plus copper groups will be conducted at a later date.

Three different raters analyzed the brain slices using high resolution images of the tissue and imageJ to quantify the plaque load of each mouse in square millimeters. The imaging software was calibrated using an objective micrometer to ensure accurate measurements. A reliability analysis utilizing intraclass correlation coefficients (ICC's) was performed to ensure that there was agreement (ICC = .71) and consistency (ICC = .84) between the raters and then an average of the three data sets was used in the final analysis. The slices were analyzed with both light microscopy and a polarized lens to check for birefringence. The regions of interest included the ventral, dorsal, and anterior hippocampus along with two areas of the basal ganglia, which can be seen in Figure 6. GLM's were conducted to compare the plaque load (DV) of the lab and iron water groups (IV's) and linear regressions were used to assess the association between freezing behavior (DV) and plaque load (IV in square millimeters). The goal was to find out what associations existed between plaque load and both water type and freezing behavior.

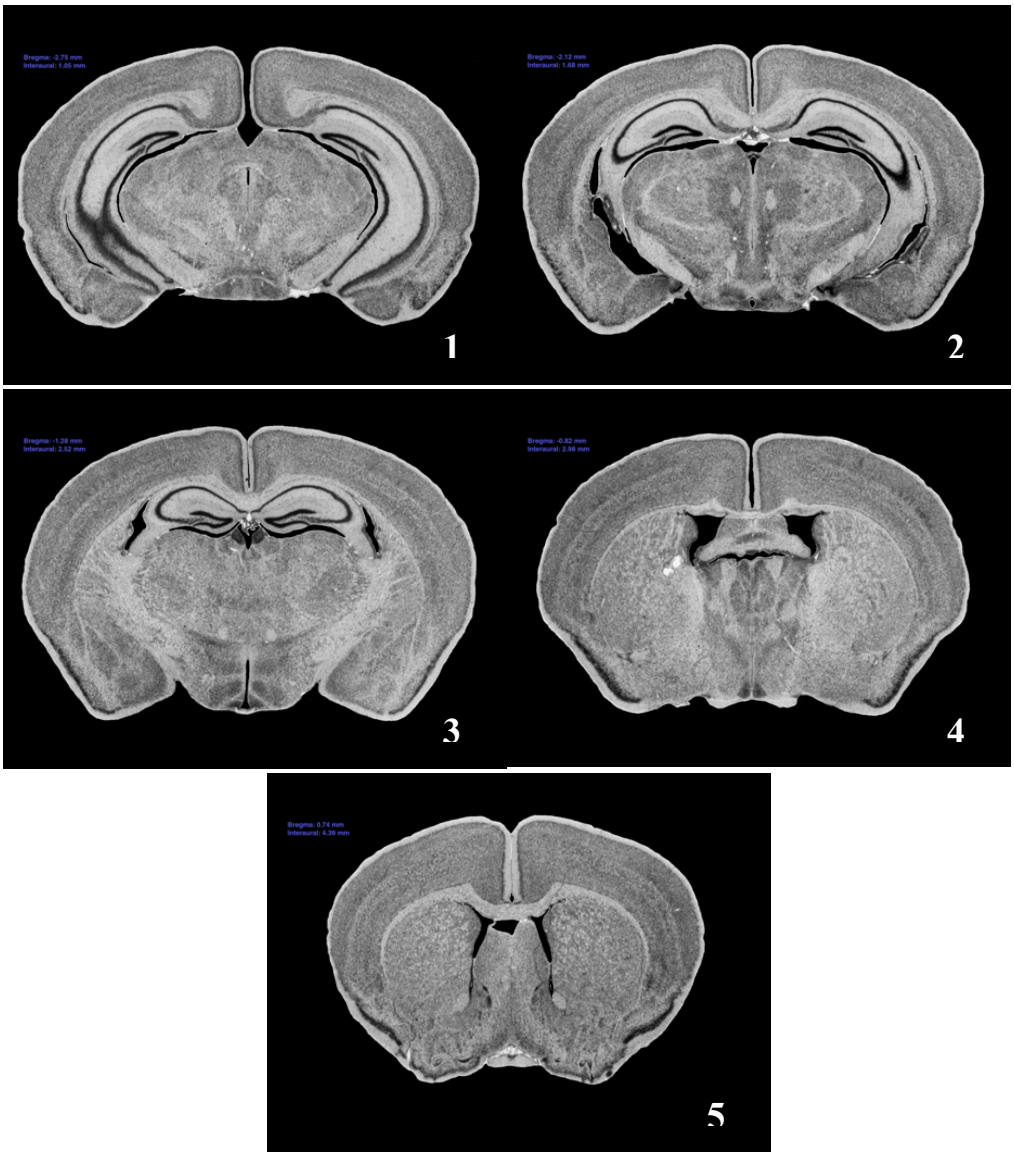


Figure 6. These are the regions of interest for the plaque study: **1**Ventral H. **2**.Dorsal H. **3**.Anterior H. **4**.Basal Ganglia (1) **5**.Basal Ganglia (2)

3. Results

Repeated Measures ANOVA's

The average percent freezing for the two genotypes can be seen numerically in Table 3 (along with their standard deviations), graphically across days in Figure 7, and across minutes in Figures 8 and 9. For Figure 7, the data have been separated into the two environments (contextual and cued) and the six minute trials have been split into the first and last three minutes; each subset of time has been averaged. For Figures 8 and 9, each day is represented for each environment across all six minutes. The habituation day was left out on all figures. Day 1 was the training day when all mice received a shock. Days 2-5 were testing days. On the training day (Day 1), minutes 123 occurred prior to the shock and minutes 456 occurred after the shock. Repeated measures analyses were performed both across minutes and across days as a preliminary analysis of the results before beginning the IGC analysis. For minutes 123 of the training day (Day 1) there was no difference between groups, but there was a significant difference for minutes 456 of the training day with the Wt mice freezing significantly more than the Tg mice ($F_{(1, 70)} = 97.8$, $p < .001$). There was also a significant difference for minutes 123 in the contextual environment ($F_{(1, 64)} = 20.192$, $p < .001$) and 456 ($F_{(1, 64)} = 38.378$, $p < .001$)

for Days 2 through 5. For the results of the contextual environment across days and minutes, the Wt mice had a larger freezing reaction than the Tg mice. While the magnitudes of the reactions were different, the two genotypes still maintained a similar pattern of behavior for the contextual environment. For the cued environment, however, the Wt mice had a smaller freezing reaction than the Tg mice for Days 2 through 4. There was a significant difference for minutes 123 ($F_{(1, 64)} = 44.429$, $p < .001$) and 456 ($F_{(1, 64)} = 33.899$, $p < .001$) in the cued environment. These results across days (Figure 7) were mirrored in the results across minutes (Figures 8 and 9). No significant difference was found between water groups and so the data were examined for genotype differences.

Table 3. Mean Percent Freezing Values

	Genotype					
	Wildtype			Transgenic		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Train123	8.66	36	5.71	12.04	36	8.58
Train456	53.14	36	15.86	20.11	36	12.25
Day1cont123	62.51	36	19.81	35.17	36	23.75
Day1cont456	69.35	36	20.38	39.11	36	20.25
Day2cont123	49.47	36	20.44	25.61	36	17.40
Day2cont456	52.30	36	22.51	30.59	36	19.26
Day3cont123	39.32	36	19.36	19.05	36	13.73
Day3cont456	40.02	36	19.60	24.44	36	16.71
Day4cont123	27.36	36	18.89	16.12	36	13.77
Day4cont456	33.25	36	17.87	22.45	36	13.57
Day1cued123	7.77	36	8.72	26.02	36	16.85
Day1cued456	24.63	36	20.21	64.98	36	25.10
Day2cued123	7.07	36	7.40	20.53	36	12.74
Day2cued456	21.39	36	15.96	51.77	36	22.73
Day3cued123	7.05	36	7.30	16.91	36	12.04
Day3cued456	13.71	36	13.83	44.67	36	24.59
Day4cued123	5.27	36	5.67	15.18	36	10.92
Day4cued456	11.88	36	10.81	35.41	36	22.38

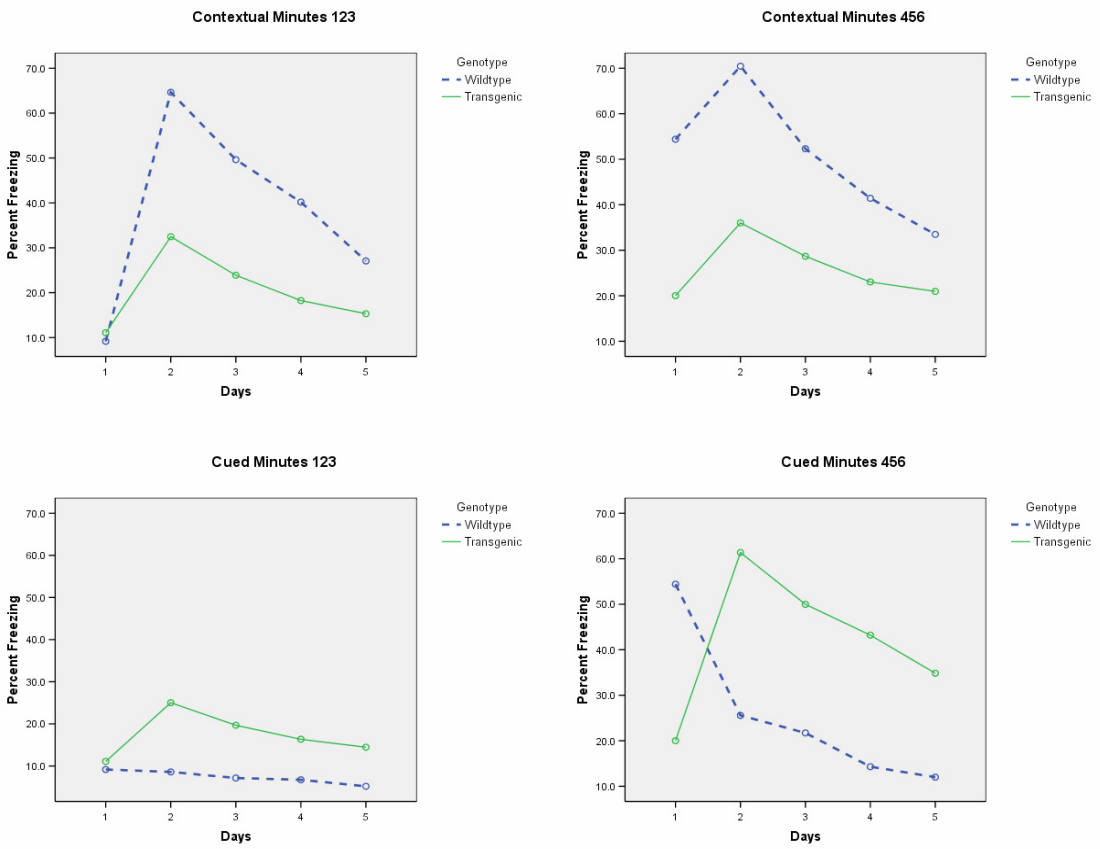


Figure 7. Means of freezing for the genotype groups (Wt and Tg) across days. The genotypes are significantly different for all days except Minutes 123 of the training day (day 1).

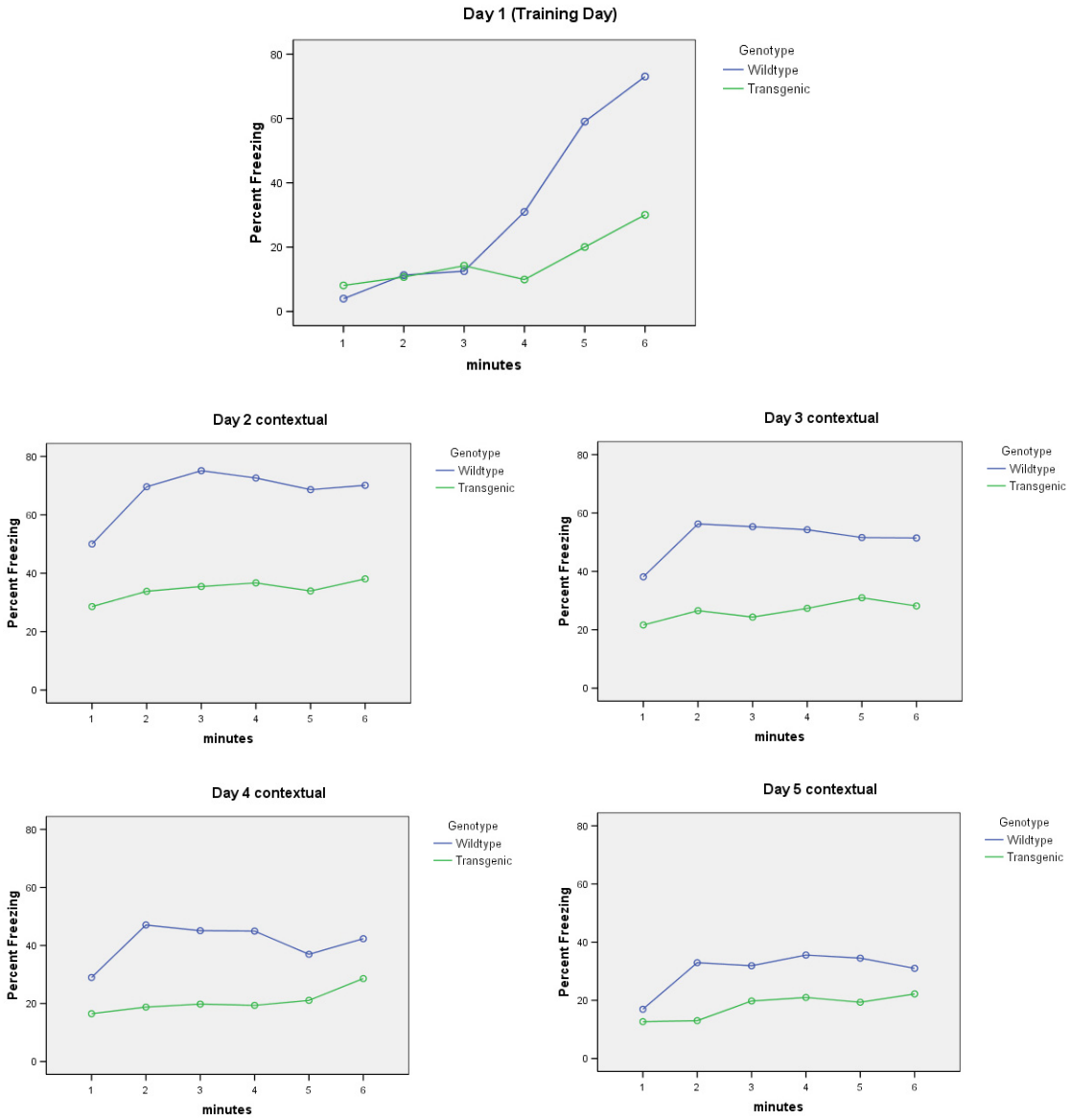


Figure 8. Means of freezing for the genotype groups (Wt and Tg) across minutes. The genotypes are significantly different for all days except Minutes 123 of day 1.

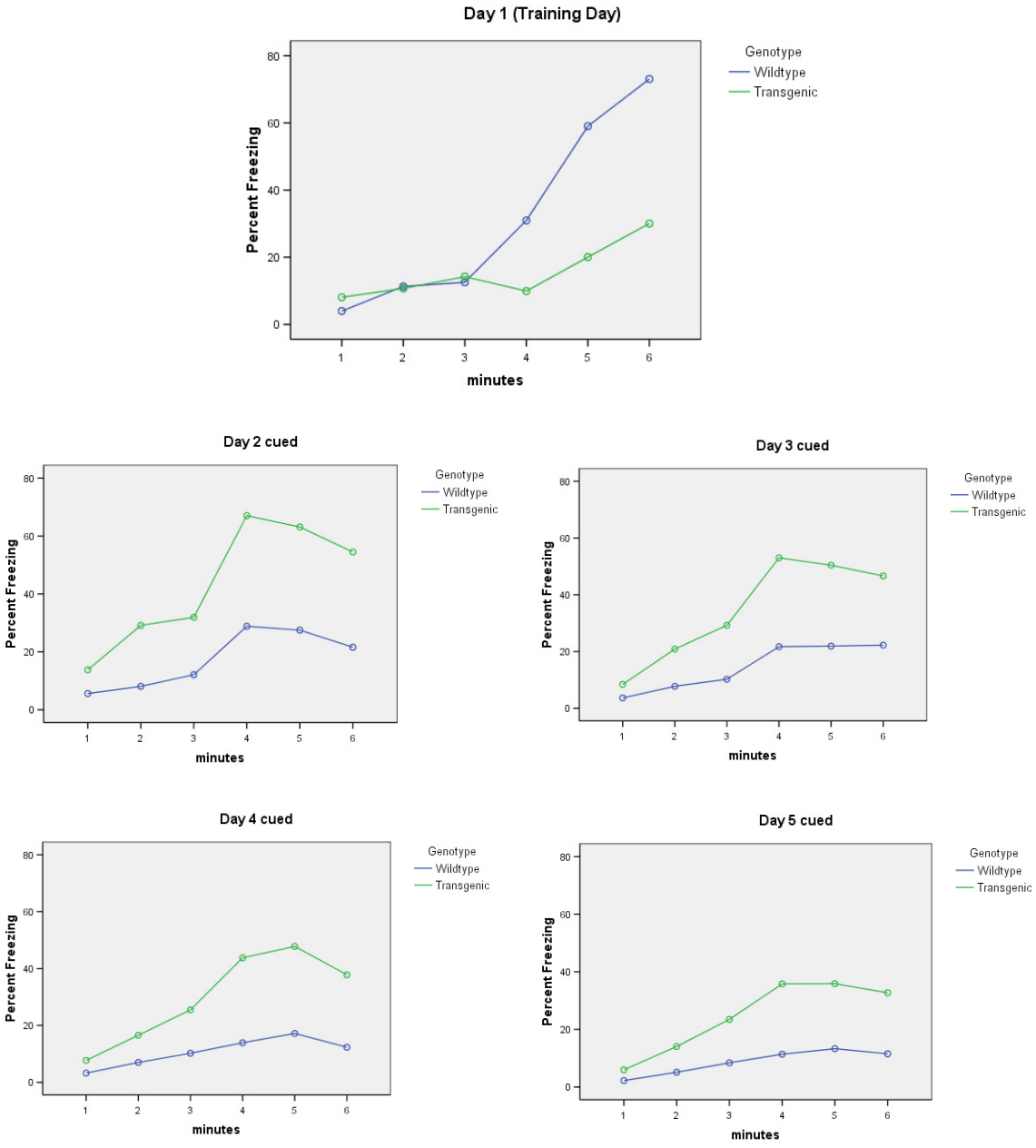


Figure 9. Means of freezing for the genotype groups (Wt and Tg) across minutes. The genotypes are significantly different for all days except Minutes 123 of day 1.

Individual Growth Curves

The data were separated into contextual and cued environments, as well as time segments of minutes 123 and 456. There were therefore results from four separate GLM's for each of these two data sets (baseline and linear). Figure 10 is comprised of graphs for the baseline and linear parameters of the contextual environment. Figure 11 is comprised of graphs for the baseline and linear parameters of the cued environment. The baseline graphs are estimates based on the Level 1 model, which is the hypothesized trajectory of the DV (percent freezing). The estimates serve as a baseline freezing measure prior to the beginning of each three minute time period. As seen in the top two graphs of Figure 10, there was a significant difference for both the first three minutes ($F_{(1, 64)} = 8.41$, $p = .005$) and last three minutes ($F_{(1, 64)} = 21.98$, $p < .001$) of the contextual environment. The Wt mice had a significantly higher baseline freezing measure for both time periods. As seen in the top two graphs of Figure 11, there was a significant difference for both the first three minutes ($F_{(1, 64)} = 17.55$, $p < .001$) and last three minutes ($F_{(1, 64)} = 21.35$, $p < .001$) of the cued environment. The Tg mice had a significantly higher baseline freezing measure for both time periods. There was no significant difference between the water types.

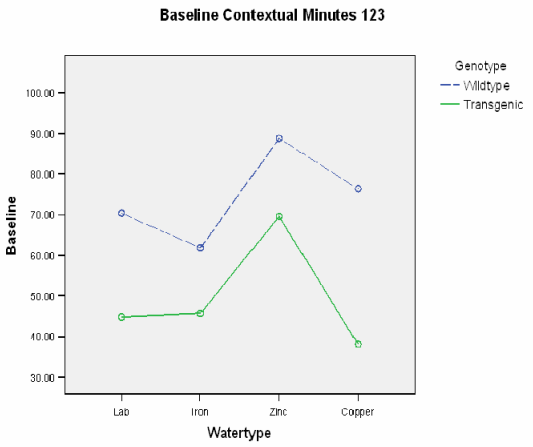
The linear parameter is the constant rate of change in freezing behavior over trial days. The higher the number, the larger the slope and thus the more the freezing behavior changed at a constant rate over the trial days. If the number is negative, the slope is negative and the freezing behavior is now decreasing at a constant rate over time. For contextual (Figure 10), the Tg mice had a greater negative slope (faster rate of change)

than the Wt for the first three minutes ($F_{(1, 63)} = 22.01$, $p < .001$) and last three minutes ($F_{(1, 63)} = 11.14$, $p = .001$). The Tg freezing behavior was decreasing at a faster rate. For cued (Figure 11), however, the Wt mice had a greater negative slope (faster rate of change) than the Tg for the first three minutes ($F_{(1, 63)} = 13.69$, $p < .001$) and last three minutes ($F_{(1, 63)} = 27.58$, $p < .001$). The Wt freezing behavior was decreasing at a faster rate. No significant differences were seen between water types.

The quadratic parameter is the change in acceleration, indicating a change in the consistency of the slope over time. There were no significant findings for the quadratic parameter ($p < .10$) for either genotype or water type.

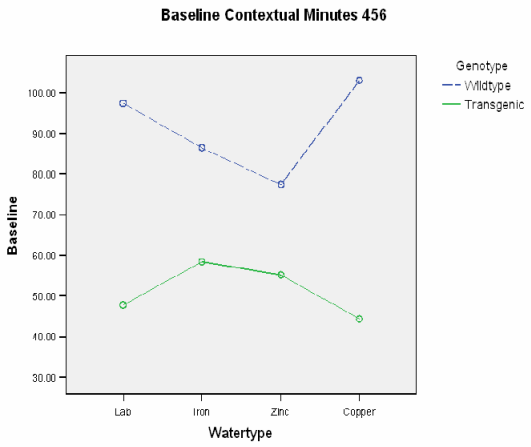
Baseline Contextual

F=8.41, p=.005



Min 123

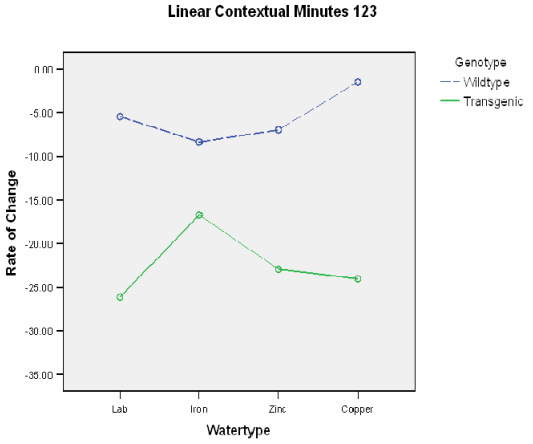
F=21.98, p<.001



Min 456

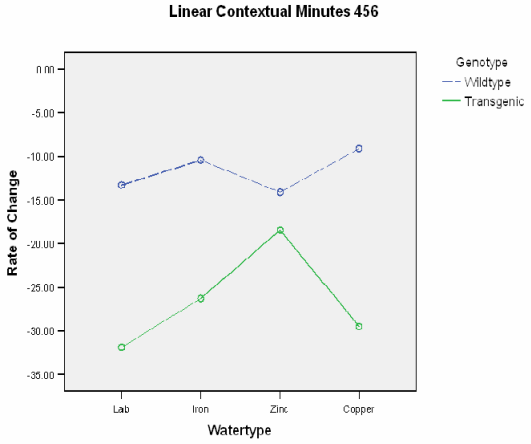
Linear Contextual

F=22.01, p<.001



Min 123

F=11.14, p=.001

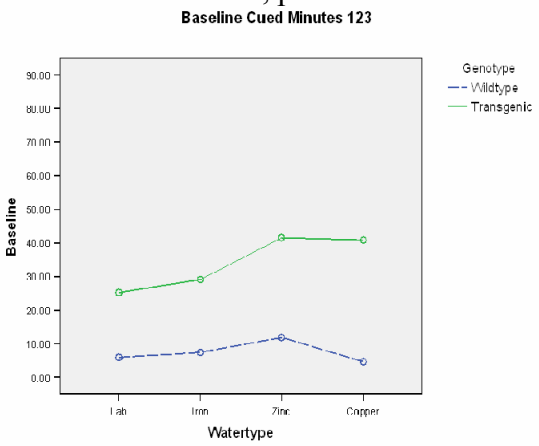


Min 456

Figure 10. The intercept represents the baseline measure of freezing behavior. The linear parameter represents the rate of change of freezing behavior. In the contextual environment for days 2-5, the Tg mice were significantly lower in baseline freezing for both time periods, but their rate of change across days was significantly greater and more negative. This suggests that the Tg mice exhibited impaired contextual conditioning compared to the Wt mice. See Figure 8.

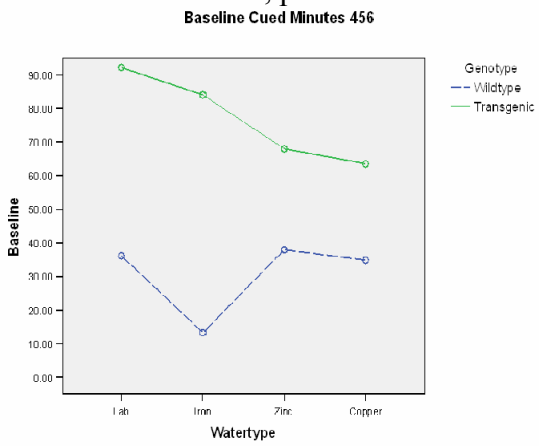
Baseline Cued

F=17.55, p<.001



Min 123

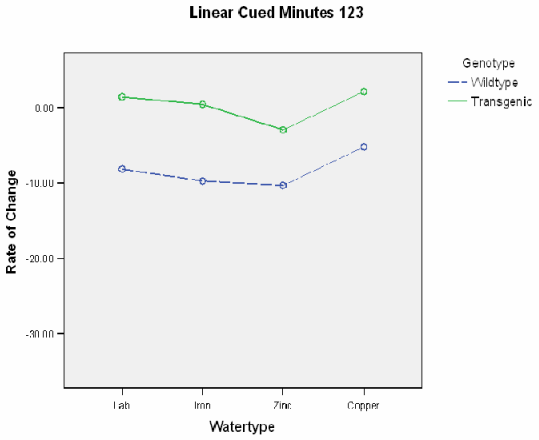
F=21.35, p<.001



Min 456

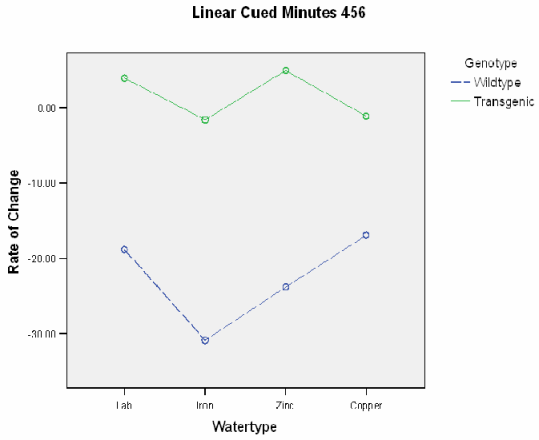
Linear Cued

F=13.69, p<.001



Min 123

F=27.58, p<.001



Min 456

Figure 11. The intercept represents the baseline measure of freezing behavior. The linear parameter represents the rate of change of freezing behavior. In the cued environment for days 2-5, the Tg mice were significantly higher in baseline freezing for both time periods, but their rate of change across days was significantly less and closer to zero. This suggests that the Tg mice exhibited enhanced cued conditioning compared to the Wt mice. See Figure 9.

Plaque Load and Behavior Comparisons

First, a reliability analysis was performed comparing the three raters on absolute agreement and consistency on the data sets. An intraclass correlation coefficient (ICC) was used to assess the agreement (similarity of the actual values obtained) and consistency (similarity of the differences found) between the three raters. The ICC was significant at $p < .001$ for all measures and the variance was non-significant and virtually zero to the third significant digit. Table 4 lists the ICC's for all measures.

Table 4. Intraclass Correlation Coefficients for the average measures of 3 raters

	Light Microscopy	Birefringence
Absolute Agreement	.745	.676
Consistency	.857	.825

With the reliability of the raters' agreement confirmed at an acceptable level, the data sets were averaged and GLM's were performed to see if the plaque levels differed between the two water groups. As seen in Figure 12, there was no significant difference between the water groups for the five regions of interest except for basal ganglia (2), where the iron group had significantly less plaque area ($F_{(1, 40)} = 11.68$, $p = .001$). This finding is similar to that seen in younger Tg mice (approximately 12 months instead of 18 as in this experiment). In the younger mice, however, this difference was much more pronounced and so age is an important factor when comparing these water groups. There was also no significant difference found between the light microscopy and birefringent plaque loads.

Linear regressions were then conducted on the different data sets to assess how brain area could predict freezing behavior. Due to the small groups in the plaque study, significance was set at $p<.10$.

No significant difference was found in behavior between the two water groups except for minutes 456 of Day 2 in the contextual environment where the iron group froze significantly more than the lab group ($F_{(1, 158)} = 9.8$, $p = .002$). In addition, a higher plaque burden was associated with a lower freezing response (negative correlation). Table 5 lists the significant results for the various brain regions on minutes 456 of the training day (Table 5a) and minutes 456 of Day 2 in the contextual environment (Table 5b). A result not listed in the table is the Birefringent ventral hippocampus plaque load predicted 18% of the freezing behavior on cued minutes 123 of Day 5 ($F_{(1, 17)} = 3.846$, $p = .066$; $r = .43$, $R^2 = .185$) and 15% of the freezing behavior on cued minutes 456 of Day 5 ($F_{(1, 17)} = 3.035$, $p = .10$; $r = .39$, $R^2 = .151$).

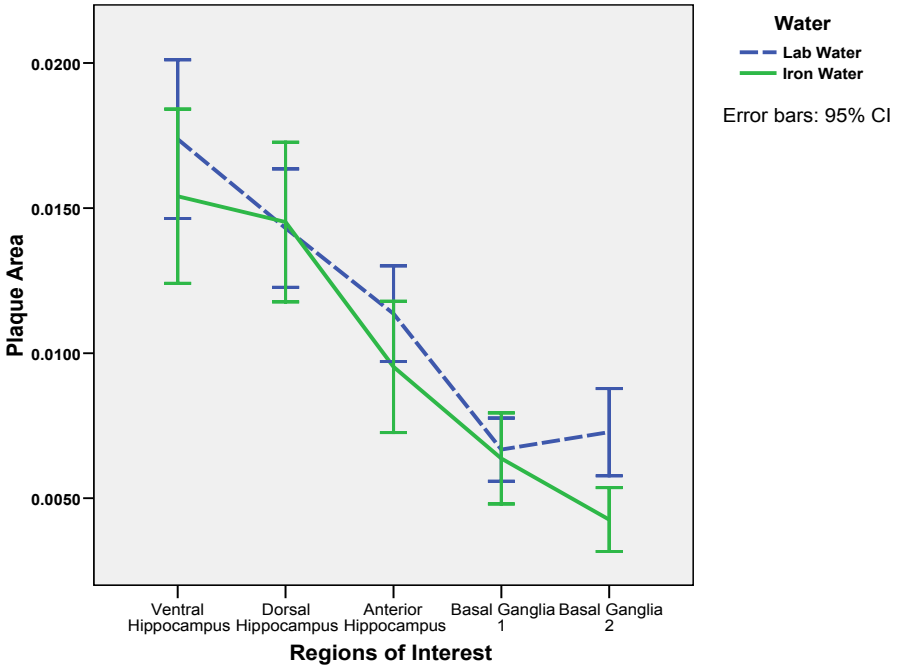


Figure 12. Comparisons of plaque load between the 5 regions of interest

Table 5. Linear Regression results for the plaque analysis

a. Minutes 456 of the Training Day (Day 1)

Area	Light	$F_{(1, 38)}$	p	r	R^2
Ventral Hippocampus	Normal	3.01	.100	-.38	.143
Dorsal Hippocampus	Normal	4.01	.066	-.44	.191
Anterior Hippocampus	Normal	3.97	.055	-.31	.095
Dorsal Hippocampus	Biref.	5.58	.030	-.49	.237
Anterior Hippocampus	Biref.	7.58	.009	-.41	.166

Table 5 (continued). Linear Regression results for the plaque analysis

b. Minutes 456 of Day 2 in the Contextual Environment

Area	Light	$F_{(1, 38)}$	p	r	R^2
Dorsal Hippocampus	Normal	6.35	.022	-.52	.272
Anterior Hippocampus	Normal	4.51	.040	-.33	.106
Basal Ganglia	Normal	5.21	.028	-.35	.120
Dorsal Hippocampus	Biref.	6.06	.025	-.51	.263
Anterior Hippocampus	Biref.	6.34	.016	-.38	.144
Basal Ganglia	Biref.	5.36	.026	-.35	.124

4. Discussion

Given the abnormal freezing behavior of the Tg mice on the training day, IGC was determined to be the best way to analyze the data. One of the major hypotheses was that water type would make a difference in the behavioral outcome. Even with the use of an IGC analysis, which focuses on individual growth while allowing baseline as well as other growth parameters (like slope) to vary, no significant difference between the four water groups could be found. This could be for a number of reasons. Perhaps the dose of metals given to the mice was insufficient to produce results in this case. Also, it could be that the advanced age of the mice or the small N values (in part due to blindness discovered in some of the animals) played a role. There may have been a detectible difference between the water groups at a younger age. The most plausible explanation is that the behavioral test used was not a valid measure because the strong differences between genotypes were overshadowing the differences between water groups. The discussion will now focus on the differences in behavior between the genotypes.

A major and unexpected result was that the genotypes were significantly different from each other for the training day. This in itself suggests that the way they behave during this particular test is fundamentally different. In this case the Tg mice are

abnormal because, as confirmed by observation and data analysis of the behaviors, the Tg mice responded with increased motion as well as freezing. This suggests that the fear reaction of the two genotypes is different. Freezing is assumed to be the normal response that mice exhibit when confronted with a fear stimulus. Without this normal response from all groups, the task has no foundation from which to test and compare different groups. This could be due to hyperactivity in the Tg mice.

The Repeated Measures ANOVA's clearly showed that the Tg mice exhibited a lower level of freezing in the contextual environment. If a reduction in freezing behavior indicates impairment in the memory of the context, then this could be interpreted as hippocampal damage.

In the cued environment, the Tg mice displayed enhanced freezing behavior. This could indicate an additive effect between the contextual and cued environments. If the animals were unable to distinguish between the contextual and cued environments, the freezing behavior in the cued environment would represent both contextual and cued conditioning. If so, this would explain why the Tg freezing behaviors were higher in the cued environment and support the theory that hippocampal damage adversely affected the ability of the mice to both acquire contextual conditioning, and distinguish between different contexts.

By looking at the intercept and linear parameters (Figures 10 and 11) from the IGC analysis, that focuses on the days after the Training Day, it's clear that the two genotypes behaved differently in the two environments. In the contextual environment, the Wt mice began each time period with a higher rate of freezing and decreased this

behavior at a slower rate than the Tg group (Figure 10). This is not clearly seen in the results from a repeated measures analysis and an IGC analysis is more useful. Repeated measures focuses on means of the different groups and by utilizing IGC's, each individual mouse's change over time has been incorporated to form a more accurate representation of how the groups change over time. This suggests that the Wt contextual conditioning was more successful and that the Tg group experienced faster extinction as indicated by a significantly greater negative linear component (Figure 10).

In the cued environment (Figure 11), however, the Tg mice began each time period with a higher rate of freezing but decreased this behavior at a much slower rate than the Wt group, which was close to zero. This suggests that the Tg conditioning to the cue was more successful and that their extinguishing was impaired. Possible explanations are amygdalar damage, masked cued conditioning, or the additive theory previously mentioned.

Another possible reason that the Tg mice exhibited enhanced cued conditioning is that due to the intensity of the shock, a second stimulus other than the tone was paired with it (such as the cameras or light sources) and if it was something constantly present in the environment, this could explain their behavior. Perhaps the shock was too intense; it has been shown that as the intensity of the US increases, mice are more likely to become conditioned to a wider range of stimuli in the environment (Phillips and LeDoux, 1992).

The possibility of an additive effect discussed above between the contextual and cued environments was examined further. If the Tg mice were unable to distinguish between the two contexts, then they would be reacting to both contextual and cued

conditioning when in the cued environment. Figure 13 shows a comparison of Cued Minutes 456 as it was originally observed in the experiment (left) and then again with the contextual value of minutes 456 subtracted from the original cued value for the Tg group (right). As clearly shown, the significant differences between the two genotypes are completely eliminated by this procedure. This supports the theory that the Tg mice are unable to distinguish between the environments and are exhibiting freezing behavior based on both contextual and cued associations while in the cued environment. The lower freezing behavior of the Tg mice in the contextual environment does not, by itself, prove that the Tg mice exhibited impaired contextual conditioning due to a cognitive deficit. The results from the additive effect, however, coupled with this lower freezing in the context strongly supports the view that these Tg mice were indeed cognitively impaired and that this impairment was most likely in the hippocampus.

Hippocampal impairment, particularly in the dentate gyrus in mice, has been shown to cause deficits in the discrimination between two similar contexts (McHugh, Jones, Quinn, Balthasar, Coppari, Elmquist, et al., 2007). However, repeated exposures to these environments did show improvement in this discrimination. This suggests that multiple training days could ameliorate the deficit.

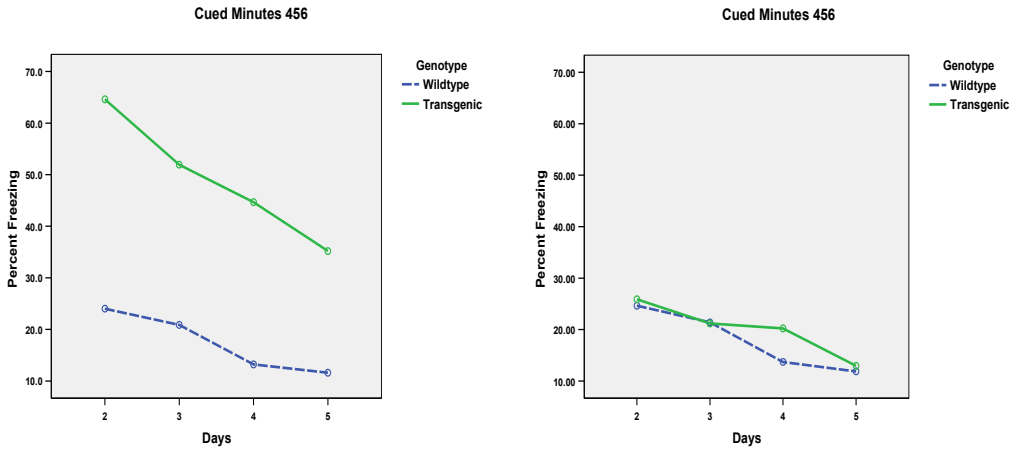


Figure 13. The differences seen between the genotypes in Cued Minutes 456 ($F_{(1, 64)} = 33.899$, $p < .001$) are eliminated once the values for the Tg contextual minutes 456 are subtracted from the Tg cued minutes 456 values ($F_{(1, 64)} = .212$, $p = .65$).

Another factor that could be influencing the low Wt freezing behavior in the cued environment is advanced age. A study by Gould and Feiro (2005) showed that cued, but not contextual, fear conditioning was impaired in mice of advanced age (19-20 months) when the time between training and testing was greater than 24 hours. In this study, the time between training and testing was indeed greater than 24 hours, so perhaps both the Wt and Tg mice had difficulty being conditioned to the tone due to the natural effects of aging. It could also be, however, that this slower extinguishing is a part of the normal response of these Tg mice and that it is simply different from the Wt mice for reasons other than amyloid presence or hippocampus/amygdala damage.

For humans, it has also been shown that healthy aging can affect fear conditioning performance as well as AD pathology. People of advancing age can exhibit a lack of arousal to an unconditioned stimulus when compared to younger groups. Also, there can

be a lack of awareness of the association between the CS and the US (LaBar, Cook, Torpey, and Welsh-Bohmer, 2004). If the older cohort exhibits a lower unconditioned response and does not learn associations as quickly as the younger cohort, this does not mean that the older cohort suffers from AD. They are experiencing changes that are associated with normal aging. In the same way, if an older cohort of mice exhibits a limited response to cued conditioning, this could be the result of advanced age. More CS-US pairings could eliminate this problem.

As a further indication of differences not only between Tg and Wt mice, but also between mouse subgroups in the Tg group itself, the plaque study found that when the Tg animals were shocked, there was a significant difference between the freezing behavior of iron and lab water Tg groups based on the level of plaque burden on minutes 456 of the Training Day and Day 2 in the contextual environment. The lab water group froze less. During the same time period of Day 2 in the contextual environment, there was a significant difference between the iron and lab water groups for plaque burden in the basal ganglia. Higher plaque burdens were found in the lab water group. A linear regression analysis showed that during minutes 456 of the Training Day and minutes 456 of Day 2 in the contextual environment, mice with more plaque area responded to the shock with less freezing behavior and vice versa (See Table 5). This could be due to hippocampus or basal ganglia damage adversely affecting the natural fear response or leading to the hyperactive behavior often observed in these animals and also suggests that water type can have an effect. The baseline response to the shock and the first measure of contextual memory could therefore be altered by the levels of plaque already present in

the brain. If there is a fundamental difference in the neurological processes of not only the two genotypes, but within the Tg group itself, it could be masking the behavioral changes that were due to water type.

Another genotype problem that came up involved the presence of a gene coding for blindness in some of the mice. These mice were incorrectly included in the groups and ten had to be removed from the study after genotyping confirmed that they had the gene for blindness. This was discovered after the completion of the study and further reduced the N values. In a study involving auditory and visual cues, blind animals can definitely skew the results. Also, there were issues with a misclassification of the genotype of some of the mice. The data was reanalyzed after they were placed in the correct group. Another issue was that the mice were tested in the FC paradigm at 14 months of age and sacrificed at 18 months of age after other behavioral tests were performed. In order to correlate plaque finding with behavior, the animals should be sacrificed immediately following a behavioral task. One final criticism on the plaque analysis involves the use of square millimeters as the measure. If this were converted to a percentage of the total brain area, it may more accurately represent the true relationship between plaque load and behavior. This is due to the fact that not all slices have the same total area and a ratio would take this potential confound into consideration.

Despite the problems, a significant correlation was found between plaque burden and freezing on minutes 456 of Day 1 and in the contextual environment of Day 2. This is one of the few correlations between plaque load and behavior that have been found.

All of these results rely on the assumptions that Tg mice have a high amyloid burden in their hippocampus that adversely affect their ability to perform in contextual fear conditioning and that a lower measure of freezing behavior indicates this hippocampal damage and cognitive impairment. While the Tg groups did freeze less than the Wt groups in the contextual environment, their freezing was far from zero. This can be explained by the use of an alternate learning strategy. It's possible that these mice do indeed have a severely damaged hippocampus and are thus unable to utilize a contextual or configural learning strategy. They can still, however, fall back on the cued or elemental learning strategy in which they focus on a stimulus in the context to utilize their intact cued conditioning abilities (Gerlai, 2001; Gerlai, 1998; Maren, Aharonov, and Fanselow, 1997). Large, dark objects have been observed as salient cues for mice and in this study the cameras, speakers, and light fixtures in the fear conditioning boxes were all black objects roughly the size of the mouse. It is highly conceivable that they could have been conditioned to one of these objects.

Previous studies have shown that rats with a lesioned hippocampus have severe to total impairments in contextual conditioning (Phillips and LeDoux, 1992). This is not the case with Tg mice that do show some contextual conditioning and there are two possible explanations. The first is that often in these studies with rats there is damage not only to the hippocampus, but the surrounding brain tissue and pathways as well. With such brain damage, it's not surprising that there is significant impairment in contextual conditioning. The second reason is that often with these rat studies the hippocampus is lesioned after the training portion of the fear conditioning task. Since the rats had a functional

hippocampus for training, they used their contextual learning strategy. Once they had been lesioned, the learning strategy did not work and they did not perform well on the task. Since Tg mice have hippocampal damage due to amyloid peptides, they are unable to utilize the contextual strategy at all and thus automatically use the cued strategy (or some other strategy) to learn the association. In this way they can still perform in the contextual conditioning task (Cho, Friedman, and Silva, 1999; Gerlai, 1998; Gerlai, 2001; Maren et al., 1997).

In order to combat this second strategy for contextual conditioning, it's possible to use a very salient cue to reduce the ability of the animal to focus on another cue in the environment. An example would be a very loud tone. If the animal has difficulty focusing on anything but the tone, the chances of using their cued strategy is lessened. Therefore, any contextual conditioning is more likely to be to the environment instead of one particular cue in the environment (Cho, Friedman, and Silva, 1999; Gerlai, 1998; Gerlai, 2001). Another method to combat this second strategy is to decrease the salience of any cues in the contextual environment. Corcoran, Lu, Turner, and Maren (2002) found that Tg2576 mice acquired contextual conditioning normally in their first experiment, but when they removed salient cues from the context, their ability to remember the context was impaired while their ability to remember the tone/shock association remained at a normal level. They concluded that the amygdala was undamaged and that the mice were utilizing their cued strategy to learn fear behaviors in the context. By removing that ability, their fear behaviors significantly decreased in the

context (Corcoran et al.). Ideally, a fear conditioning paradigm would utilize a very salient cue with no other salient cues in the context.

This explains the fallacy of the first assumption that mice need a hippocampus to perform in all contextual conditioning situations. The second assumption is that reduced freezing always indicates a cognitive deficit. Gewritz, McNish, and Davis (2000) found that hippocampal lesions actually disrupted the expression of freezing behavior in rats. Using a separate method of measurement, they showed that contextual conditioning had occurred, even though levels of freezing in the contextual environment remained low. Their contextual blocking method showed that a prior contextual conditioning event reduced the conditioning of the rats to a tone later on. The limited cued conditioning was used as proof that conditioning to the context had taken place earlier and had taken precedence over future conditioning. They also used the same rats to test their freezing behaviors to natural US's without contextual conditioning. They found that the freezing response was consistently lower in the lesion group, further supporting their hypothesis that damage to the hippocampus can adversely affect the natural fear response of freezing in rats (Gewirtz et al.).

Hyperactivity, an additional effect seen in Tg2576 mice with hippocampal damage, may explain the reduction in the initial freezing behavior of these mice during the Training Day (Day 1). As previously mentioned, these mice have shown hyperactivity in a number of behavioral tests (Ognibene et al., 2005) and this was supported by observations made during the course of this study, both in their home cage and in various behavioral testing environments. Although Tg2576 mice exhibit

hyperactivity, not all transgenic mice with hippocampal damage do. Lalonde, Dumont, Staufenbiel, Sturchler-Pierrat, and Strazielle (2002) showed that the APP23 transgenic mouse, that also has the Swedish mutation, had fewer ambulatory movements. This mouse differs from the Tg2576 in that it does have microglial reactivity and hyperphosphorylated tau proteins.

The overall conclusion is that when looking at the effects of water type on freezing behavior, the strong differences found between the genotypes could have overshadowed weaker differences between water types that may have been present. There were many differences in the way the genotypes reacted to the fear stimulus and when a fear response is the measure being studied with a behavioral test, it is difficult to accurately compare the memory of a fear stimulus in two groups with different fear reactions. A lack of a change in behavior across time in this case could indicate a loss of memory, or could simply mean that the memory in question is not linked with a particular target behavior pattern in certain groups of mice. Therefore, we cannot assume that an absence of freezing in the Tg mice is an absence of memory. When comparing behavior patterns of two groups of genotypically different mice, the normal fear conditioning paradigm will not always work. Such is the case here. A different paradigm will be necessary if minute changes in memory and behavior are to be detected in the different metal groups.

Summary

Several important discoveries were made during the course of this study including the surprising difference between the fear responses of the genotypes on the Training Day. The lower freezing response of the Tg mice was unexpected and was resolved through the use of IGC analyses. It was also demonstrated that Tg mice exhibited impaired contextual freezing with an inability to distinguish between the environments. Other important conclusions were the negative correlations between freezing behavior and plaque burden, the discovery that water type can have an effect on plaque deposition in certain parts of the brain (basal ganglia), the association between plaque deposition and changes in motor behavior such as hyperactivity, and the influence this hyperactive behavior can have on the freezing behavior of the Tg2576 mouse model of AD.

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Curriculum Vitae

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