THE BEHAVIORAL EFFECTS OF ZINC ON MICE EXPRESSING THE HUMAN P301L GENEBY

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Fairfax, VA

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by

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Bachelor of Science
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DEDICATION

This is dedicated to my loving family who have always shown interest in my work as well as to Zena who has kept me happy and sane throughout this and all of my research. Finally, this is dedicated to Franz Ferdinand the cat, my daytime writing partner.

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I would like to first thank Dr. Jane Flinn, Dr. Erin Murdoch, and Dr. Craig McDonald for their support, advice, and guidance throughout this experiment. I would also like to thank Kristen Craven for running this experiment with me and my lab mates for assisting with advice and research. Finally, I would like to thank Zena Kirby for editing this manuscript and adding hundreds of necessary commas.

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LIST OF ABBREVIATIONS

Alzheimer's disease ..... AD
Zinc Enriched Neuron ..... ZEN
Parts Per Million ..... PPM
Circadian Rhythm ..... CR
Morris Water Maze ..... MWM

ABSTRACT

THE BEHAVIORAL EFFECTS OF ZINC ON MICE EXPRESSING THE HUMAN P301L GENE

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George Mason University, 2018

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Alzheimer's disease is the most common form of dementia in the world, being responsible for between 60-70% of all cases of dementia, and is characterized as a chronic progressive neurodegenerative disorder. Patients with Alzheimer's disease display deficits in many cognitive areas such as memory, depression, and social functioning. Although a diverse number of factors have been considered to play a role in Alzheimer's disease, the pathology of neurofibrillary tangles is present in all patients with the disease. Neurofibrillary tangles form from hyperphosphorylation of the Tau protein and are responsible for the death of neurons. As tangles spread further in the brain, cognitive deficits increase as well. Another major health issue of discussion is the bodies' use of biometals such as zinc and copper. Biometals are necessary for healthy body function but must be maintained within a certain healthy range; if problems with homeostasis cause the metal to fall outside of this range, then health problems will occur.

The disruption of biometal homeostasis has been implicated in many diseases including Alzheimer's disease. The levels of zinc and copper in the body are positively related to the amount of neurofibrillary tangles. Despite the large body of work on biometals and Alzheimer's disease, no study has looked at the cognitive effects of excess zinc in a mouse model containing neurofibrillary tangles. This study examined the behavioral effects of excess zinc on transgenic mice containing the human P301L gene which produces neurofibrillary tangles in the mice. Fifty-two total mice were tested beginning at an age of three and half months old. Behavioral tests ran include a seven day Morris Water Maze paradigm as well as a seven day circadian rhythm assay and a two day nesting paradigm. Tau mice performed significantly worse than wild type mice in time to find the platform, number of platform crossings, thigmotaxis, nesting, and circadian rhythm. Deficits in nesting, circadian rhythm, and time to find the platform were significantly impaired for the transgenic mice drinking zinc water compared to transgenic mice drinking tap water. Tau mice consuming zinc water built the poorest nests and had the longest amount of time to find the platform in Morris Water Maze testing. These findings add to the growing literature on the negative role of zinc in neurodegenerative disorders including Alzheimer's disease. The elderly population may need to be cautioned about the consumption of excess zinc.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia worldwide, accounting for 70% of all dementia cases. It is estimated that there are approximately 26.6 million people living with Alzheimer's disease currently and the number of elderly individuals affected with AD is expected to quadruple by 2050 (Plassman, 2007). As the amount of elderly individuals increases, so will the amount of people diagnosed with Alzheimer's disease, which will compound the social and economic burden already faced due to the disease. The social burden includes not only the changes in memory and social function that a person suffering from the disease experiences, but also the time and stress of family and caregivers who are supporting this person. The economic burden is the healthcare costs associated with taking care of individuals suffering from the disease, a number which increases every year. Due to these burdens, it is imperative that a cure to the disease is found; however, even after many years of research, only the underlying pathology and genetics have been discovered with the cure continuing to elude researchers.

Alzheimer's disease can be divided into two forms, late onset and early onset. Early-onset AD, also known as familial AD, is a purely genetic form of AD that generally affects people who are more middle-aged (40-60). This form of AD accounts for approximately 5% of all disease sufferers. A mutation on the PS1 (presenelin) gene is

responsible for the majority of early onset AD cases (Tanzi & Bertram, 2005). Late-onset AD affects people at a later age in life (65+) and also has a genetic component, but is not fully a genetic disease in that environmental factors play a role in the risk level for getting AD. Genes that are known to be involved in Late-onset AD include the APOE gene (Corder et al., 1993). Even though early-onset and late-onset AD affect people at different times in their lives and have different genetic components, the biological and cognitive symptoms follow a similar course for both variations of the disease.

The hallmark cognitive symptom of AD is memory loss. This memory loss can present itself in a multi-faceted way affecting spatial memory, short term memory, episodic memory, causing noun aphasia, and in some cases prosopagnosia (Reisberg et al., 1987). Other symptoms of AD include disruptions in circadian rhythm, depression, and social functioning (Reisberg et al., 1987). These symptoms are a direct effect of the underlying neuropathology.

There are two neuropathies found within every case of AD, amyloid plaques and neurofibrillary tangles. Beta amyloid plaques form from the incorrect (amyloidogenic) cleavage of the amyloid protein (Glenner &Wong, 1984). These cleaved fragments of protein are not able to be cleared through the conventional mechanisms and clump together to form visible extracellular plaques in the brain. The other pathology associated with AD, neurofibrillary tangles, is produced due to the hyperphosphorylation of the Tau protein (Grundke-Iqbal et al., 1986).

In order to better understand how the Tau protein works, and why the protein's failing produces the effects it does, the role of microtubules must first be discussed.

Microtubules are a major part of the cytoskeleton of the cell and help to give cells their shape (Weingarten, Lockwood, Hwo, & Kirschner, 1975). They are involved in a multitude of basic cell functions such as movement and cell separation through mitosis and meiosis. More important to the topic at hand, they form the structure through which intracellular transport happens (Weingarten et al., 1975). One example of this transport is the movement of both organelles and vesicles from the cell body of neurons to the pre-synaptic terminal. These microtubules are stabilized by microtubule associated proteins.

One of these proteins is the Tau protein. The Tau protein is primarily found in neurons within the brain, although it has also been reported in both oligodendrocytes and astrocytes (Weingarten et al. 1975). Within neurons, the protein was thought to only exist in the axon of the neuron, but recently preliminary evidence may suggest Tau can be found in dendrites as well (Hoover et al., 2010). A Tau protein can be phosphorylated (have a phosphate group added to the protein) and still retain its normal function as a stabilizing association protein. This changes when the protein is hyperphosphorylated (Iqbal et al., 1986). Following hyperphosphorylation the Tau protein starts to aggregate with other hyperphosphorylated proteins to form a "tangle" (Iqbal et al., 1986). With the association proteins tangled, the microtubules can no longer successfully carry out their function and the cell loses its viability. Braak and Braak (1991) were able to directly correlate the intensity and location of tangles with the severity of the disease and separated the spread of disease into six distinct stages, known as Braak staging.

The creation of tangles and the behavior symptoms associated with AD can be seen in a transgenic mouse model, the P301L model from The Jackson Laboratory (JAX).

This mouse model has a human gene responsible for Tau (P301L) overexpressed in its genome causing tangles to be seen in the brain after six months and causing deficits in memory found through tests, such as the Morris Water Maze, after four months. Before talking about the tests to be run in this experiment, a second topic is important to consider: bio-metals and their homeostasis.

Bio-metals such as copper, Iron, and zinc all play a major role in the body and were originally found in bones and muscles. It became known in the years following these initial discoveries that the role also extended to the brain. Although the majority of discussion on chemical communication in the brain focuses on complex molecules such as acetylcholine and glutamate, bio-metals play an invaluable role in both the body and brain (Frederickson, Koh, & Bush, 2005). Without metals such as copper, Iron, and zinc, which make up a miniscule amount of our total body mass, we would not survive. For the purpose of this paper, only the role of zinc in the brain is discussed.

Zinc has the second highest trace metal concentration in the human body after Iron, weighing in at between 3 and 4 grams (Takeda, 2001). Of this amount the majority (90%) is found in the muscles and bones of the body, and in males there is a large concentration in semen and the prostate gland. Of the 10% in the brain, zinc is located in multiple regions and serves multiple functions, with 10% of the zinc in the brain found in zinc Enriched Neurons (ZEN) (Takeda, 2001).

Zinc Enriched Neurons are found primarily in the hippocampal region as well as the amygdala (Takeda, 2001). If there is an inadequate amount of zinc in the diet, deficits can be seen in spatial memory tasks in humans. Experiments with mice and rats have

shown that a diet with heavy zinc supplementation at 10 parts per million (ppm) can cause spatial memory deficit in the Morris water maze (Flinn et al., 2005; Railey, Micheli, Wanschura, & Flinn, 2010). Given that most ZEN are found in the hippocampus this dietary effect is not surprising as the hippocampus is understood to be a vital component of spatial memory. In the vast amount of these neurons, zinc is co-released with the excitatory neurotransmitter glutamate; however, neurons have been found where zinc is co-released with the inhibitory neurotransmitter GABA (Takeda, 2001). Zinc is thought to play a role with the post synaptic receptor as a neuromodulator, but the full mechanism of interaction is far from understood (Takeda, 2001).

It is thought that bio-metals do not have a precise identifiable amount that must be present in any one area of the brain to promote healthy functioning. The balance between concentrations in different areas is thought to be more important than the concentrations themselves (Takeda, 2001). Zinc has been implicated in many neurological diseases, such as Picks disease and Parkinson's disease, and is thought to have such a profound effect on AD that one theory of AD is the "The Zinc Dyshomeostasis Hypothesis" (Craddock et al. 2012)

The role of zinc in AD has mainly been examined regarding its binding to amyloid beta (Bush et al., 1994); however, zinc also interacts with the Tau protein (Kim et al., 2011). Using molecular modeling, Craddock et al. (2012) in their work outlining the zinc Hypothesis of AD found that low or excessive levels of zinc will destabilize microtubules which will lead to neurofibrillary tangles. They theorized that this is due to disruption in side-to-side electrostatic attraction between tubulins. Kim et al. (2011) took

it a step further than the previous experiment modeling by using human neuroblastoma cells transfected with Tau in culture. They found that placing zinc into these cultured neurons caused phosphorylation of Tau at the s214 location. This was due to a Ras/Raf/MEK/ERK cascade which causes interference into the polymerization of microtubules. Sun et al. (2012) found similar findings in cultured rat brain tissue, except instead of showing that this was due to a cascade they found that this was due to the inhibition of a protein phosphatase which can be reversed by treatment with a zinc chelator.

Huang et al. (2014) used a fly model of Tauopathy which contained a mutated human version of Tau. Their study once again showed that zinc binding increases Tau phosphorylation; furthermore, they went on to show that zinc can bind directly to Tau and cause toxicity in a mechanism that is independent of phosphorylation. This finding is interesting in that it demonstrated that zinc can itself cause toxicity without having to cause phosphorylation, which is something that other studies up until this point have not shown.

All of these studies using models of Tauopathy with zinc have portrayed that zinc can facilitate the phosphorylation of Tau and foster the creation of neurofibrillary tangles. However, all of these studies also lack one very important thing: behavioral testing of zinc with Tauopathy. As important as neuropathologies are, a disease like AD is defined by its cognitive loss. How zinc interacts with the Tau protein in cell cultures is valuable information, but whether or not this interaction affects the cognitive aspects of this disease is equally important.

This experiment seeks to fill this gap in knowledge about the cognitive and behavioral effects of the role of zinc interactions with Tau. P301L/CamKII mice possess the human mutation for Tau and develop Tau and its related deficits, and compose half of the animals in this experiment, with the other half being wild type mice. Furthermore, each genotype was split in two with half of the mice receiving lab water and half of the mice receiving lab water with added zinc (10 ppm). These mice were then tested for behavioral deficits that are associated with AD: spatial deficits tested with Morris Water Maze, Circadian Rhythm (CR), and Nesting.

Hypothesis

The hypotheses of this experiment are dependent on the test:

1) Morris Water Maze: Previous evidence has shown that zinc supplemented Mice do worse than lab water mice, so we anticipate a main effect of water causing deficits in performance. Furthermore, a main effect of genotype is expected with Tau mice performing worse than wild type mice across all measures. Finally, we expect an interaction effect between genotype and water with zinc supplemented Tau mice performing the worst.
2) Nesting/CR: Mice with Tau should portray deficits in these behaviors, as these behaviors are associated with AD. Therefore, we expect a main effect of genotype with Tau mice showing deficits in comparison to wild type mice. A main effect of water is not expected as there is no ZEN in the areas of the brain responsible for these behaviors which may be disrupted. Finally, a

genotype-water interaction effect is hypothesized with Tau mice consuming zinc water performing the worst.

METHOD

Animals

Fifty-two animals were used for this experiment. Twenty-six of these mice were wild type and twenty-six were transgenic animals containing both the P301L mutation and the CamKII mutation. The CamKII mutation is important because the Cam Kinase promoter is needed in order to translate the P301L gene so that a phenotype can be seen. Each of these groups of 26 were split into 2 groups of 13, one of which received zinc enhanced water and the other of which received lab water.

Breeding, Genotyping, and Housing

Three Male mice containing the P301L mutation were ordered from The Jackson Lab (stock no: 015815, Bar Harbor, Maine), as were 9 wild type female mice on the background strain C57BL/6J (stock no: 000664). Upon arriving at our facilities the mice were separated so that each male was in his own cage and the females were housed with 3 mice per cage. All animals were given “love mash” (Bio-Serv. Flemington, New Jersey), a high fat diet that raises reproductive activity, for a period of two weeks before breeding to help foster estrous and prepare the mice for breeding. Five days prior to pairing for breeding, bedding from the male cage was placed within the females’ cage and bedding from the females’ cage was placed within the male cage. The scents from the opposite sexes help to prepare the mice for breeding. The exchanges of bedding only

happened between breeding pairs so that a male cage receives the bedding only from one female cage and vice versa. After the two weeks on the love mash, and five days after the bedding exchange the mice were paired for breeding by moving the three females from their cage into the males' cage. This created three breeding harems of 1 male and 3 females.

The mice were given ad libitum access to food, water, and love mash and remain paired for two weeks undisturbed to increase the chances of conception. After two weeks, the females were separated into individual clean cages with food and love mash. The female mice were monitored for litters for three weeks or until a litter was born, whichever came first. Female mice producing litters had their cage undisturbed for at least 5 days following birth. Once the newly born mice were between 11-21 days of age they were genotyped. This is done within this age range as the highest DNA yield is found in the tail and the mice do not yet feel pain in their tail. The procedure for genotyping is scruffing the animal and taking a small cut (~4mm) off of the end of the tail. This tail samples were then sent to Transnetyx, a company that specializes in genotyping, who analyzed the sample and determines the genotype of the mouse. The 9 mothers produced approximately 72 pups; of these 72 pups, 16 were female mice possessing the Tau mutation. These pups were kept and the rest of the mice were either humanely euthanized or given to other researchers to use in their experiments. Before post-natal day 28 these female mice were weaned and placed into cages with 5 or 6 females in each.

At this point in the study, an additional 3 males possessing the CamKII mutation (stock no: 007004) were purchased from The Jackson Lab. Upon the arrival of these three males, the breeding process occurred twice more exactly as described above. In this case the 3 new male mice were bred to the 16 female mice possessing the Tau mutation from the first breeding cycle. The only exception to the above described breeding process were that in the second round of breeding two of the males were in a harem with 2 females as opposed to three.

In total this produced over 115 mice. Of these mice: 26 possessed the CamKII mutation and the Tau Mutation (experimental group), 26 possessed neither the CamKII mutation nor the Tau Mutation (control group), and the remainder contained only one of the necessary genotypes and were not used in the experiment. To find out the exact genotype of these mice, the genotyping procedure described above was used. Following genotyping, these mice were weaned as described above and placed into cages of up to 4 males or 6 females in each cage. The exact number of mice in each cage varied due to the fact that 4 distinct groups of 13 need to be created for this experiment. (See Table 1). At approximately 6 weeks after birth the mice were being handled 3 days a week in order to familiarize the mice with human contact. Two weeks after this (at approximately 8 weeks old) the mice were started on their different water conditions as described below.

Table 1 - Animal Numbers and Conditions

	Wild Type	Tau/CamKII
Lab Water	13	13
zinc Water	13	13

General Colony Guidelines

All personnel working on this experiment were properly trained beforehand and made sure to wear appropriate Personal Protective Equipment for all procedures performed.

Food and Water

All mice had food available ad libitum throughout the experiment. This food, non-autoclaved Tekland 7012 (Harlan Laboratories) has been used in the past in the lab with no ill effects. Mice were transitioned to their experimental water conditions at 8 weeks of age. Half of the Tau mice and half of the Wild Type Mice received lab water with a further addition of 10ppm Zn carbonate. The other half were given regular lab water. Zn water was prepared using a 10,000ppm solution of Zn dissolved in 5% nitric acid. The solution was buffered using sodium carbonate ($NaCO_3$) to bring it to a pH of 7. This method of preparation has been used in the lab before and has proven to yield the correct amount of zinc (10ppm).Water samples were taken every 2 weeks and tested strictly for metal content using inductively coupled plasmaoptical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS, Reston,VA). All animals continued on these separate water conditions until the end of the study, at approximately 7 months after birth.

Behavioral Testing

Morris Water Maze (MWM)

MWM was conducted following Nesting and CR, when the mice were approximately 5.5 months old. Mice were placed in a pool with a diameter of 4 feet which contains a submerged (~5mm below surface), nonvisible, platform. Water was dyed white in order to render the platform invisible from the water's surface and dyed with non-toxic paint (Becker's School Supplies, Pennsauken, NJ). The pool was divided into four separate quadrants. At the apex of each quadrant lies a visual cue approximately a foot from the apex. These cues were black and white, each a different shape and also a different pattern in order to foster maximum difference between the shapes. Water temperature remained at a constant 26 degrees Celsius (±2 degrees, water temperature was taken daily before experiments began) and occurred over 8 consecutive days. Of these 8 days, the first six were normal test days in which the mouse had 3 trials to find the platform (max. 60 seconds) with a 45 second inter trial interval during which the mouse was back in its testing cage. The hidden platform remained in the same location for all test sessions; however, the starting locations varied from day to day, and between trials on each day. The third trial of days two, four, and six were Atlantis trials in which the platform was lowered for the duration of the trial and the number of platform crossings was recorded. The seventh day was one probe trial for each animal which tested the 24 hour memory of the mouse. On the visible platform trials (day 8) the platform was moved and above the water for two trials, these tests were done to ensure the animal does not have compromised eye sight. All recording and analysis were done using Colbourn

Instruments WaterMaze3 (Allentown, PA). The data that extracted from this test was the latency to locate the platform on test days, and the number of platform crosses and amount of time spent in the correct quadrant on Atlantis Trial Days. Separate data were collected on thigmotaxis (a measure of anxiety), which is based on how long the animals spend near the walls of the pool. For the entirety of Morris Water Maze experimentation, the mice were handled by Research Assistants who were trained and rehearsed the experimental procedures either in other experiments or with pilot animals.

Nesting

For two days the animals were placed in individual cages that contained the corncob bedding as well as short strips of paper. The mice were checked on daily for well-being but otherwise undisturbed for the two days. The normal action of a mouse is to use these strips of paper to build a nest to stay under. After five days, pictures of the cages were taken and scored by two independent observers on the level of nest built which were on a scale of 1-5 ranging from no nest built to all the paper moved to form a nest.

CR

This behavior was conducted starting 3 days after nesting. Mice were placed in individual cages for 6 days and not disturbed. The mice were monitored daily to be sure their food and water levels were high enough. The cages have running wheels which were connected to a computer running Clock Lab software which keeps a count for every time the wheel completes a rotation. The first and last day's data were thrown out as there is experimenter interference within these days and the remaining day's data was

extracted. This data shows how often the mouse was active, for how long it was active, and when the activity started and stopped over the 5 days of data collection.

Statistical Testing

All statistical analyses were conducted using IBM SPSS version 22 (Armonk, New York). Data were subjected to Levene's test of equal variances before running statistics. For all statistics the 2 independent variables are gene of mouse and water type. For all statistics Tukey post-hoc tests were performed if significance was found.

Morris Water Maze: A 2x2x6 Mixed Effects ANOVA was run on the following dependent variables: time to platform, number of platform crosses on hidden platform day, time spent in correct quadrant, and thigmotaxis.

Nesting: A 2x2 between-subjects ANOVA was performed using the dependent variable of nesting score, which was assessed by two blind observers with each mouse being given a score ranging from 1-5.

CR: A 2x2x5 Mixed Effects ANOVA was performed on the dependent variable of onset of activity.

RESULTS

Morris Water Maze - As seen in Figure 1, Tau mice performed significantly worse in their time to find the platform than did wild-type mice, $F(1, 52) = 147.101$, $p < .005$. This deficit was exacerbated for Tau mice drinking zinc water, showing a significant interaction effect of water type and genotype, $F(1, 52) = 4.314$, $p < .05$. Post-hoc testing on this interaction shows both Tau groups being significantly different from both wild type groups (ps $< .001$) and Tau mice drinking zinc water being significantly worse than Tau mice consuming lab water ($p < .05$). There was no significant difference between the wild type groups ($p > .05$). This was the only interaction effect seen for Morris Water Maze. Additional main effects of genotype were found in the measurement of thigmotaxis, $F(1, 52) = 97.337$, $p < .001$ (Figure 2) as well as the measure of number of platform crossings, $F(1, 52) = 105.393$, $p < .001$ (Figure 3). For thigmotaxis, transgenic mice were significantly more likely to spend time near the walls of the apparatus and when measuring number of platform crossings, transgenic mice had significantly less platform crossings than wild type mice. There were no significant effects seen for the measurement of percent of time in quadrant.

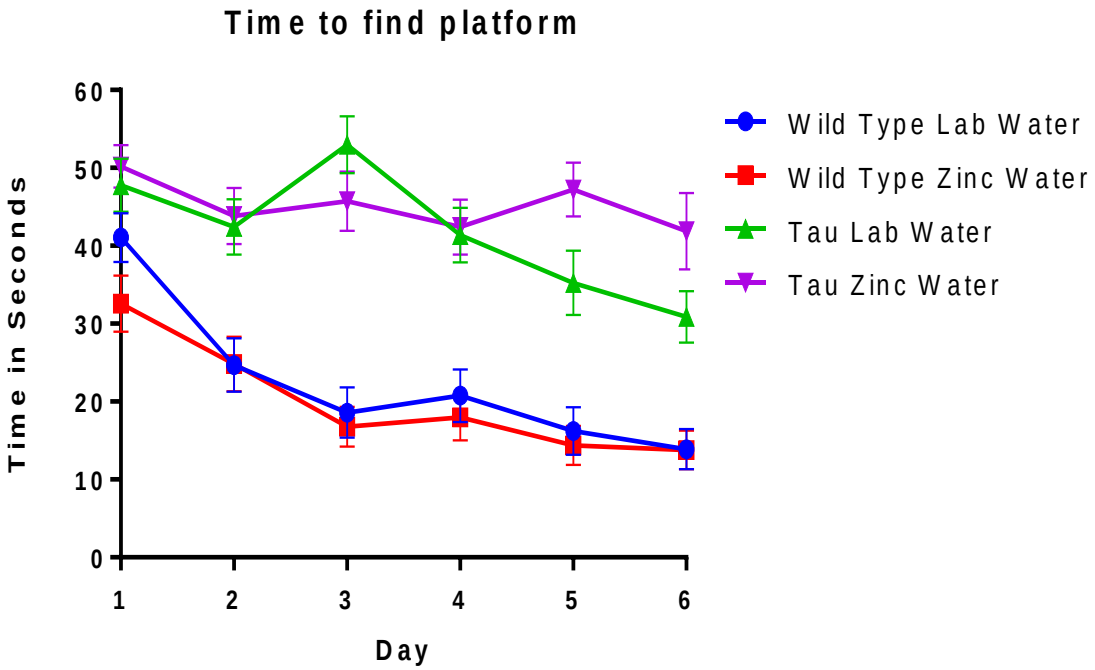

Figure 1- Average time to find the platform for each day of Morris Water Maze testing. Each day consisted of 3 trials that were averaged together. Wild type mice were able to locate the platform in less time with each subsequent day. Tau mice consuming lab water stagnated for a few days before seeing a decrease in time to find the platform and there was no decrease in time to find the platform found in Tau mice consuming zinc water. There was a significant interaction effect between genotype and water type.

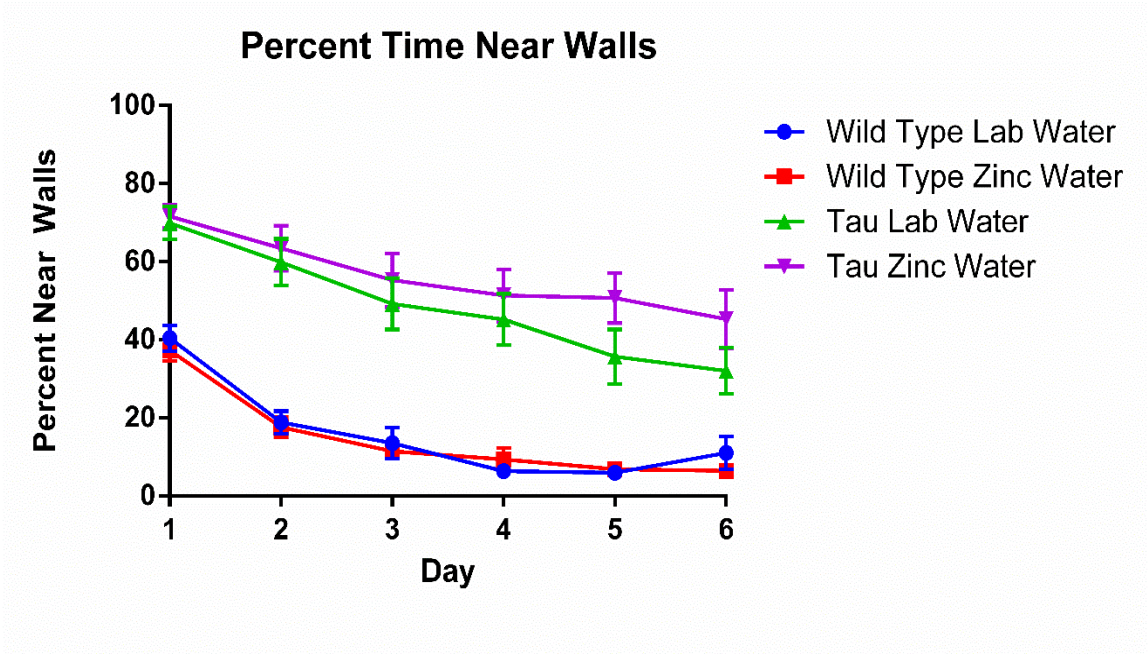

Figure 2- Amount of time spent along edges of the apparatus. Tau mice spent significantly longer along the edges of the apparatus compared to wild type mice. All mice saw a decrease in percent of time near walls over the course of the experiment.

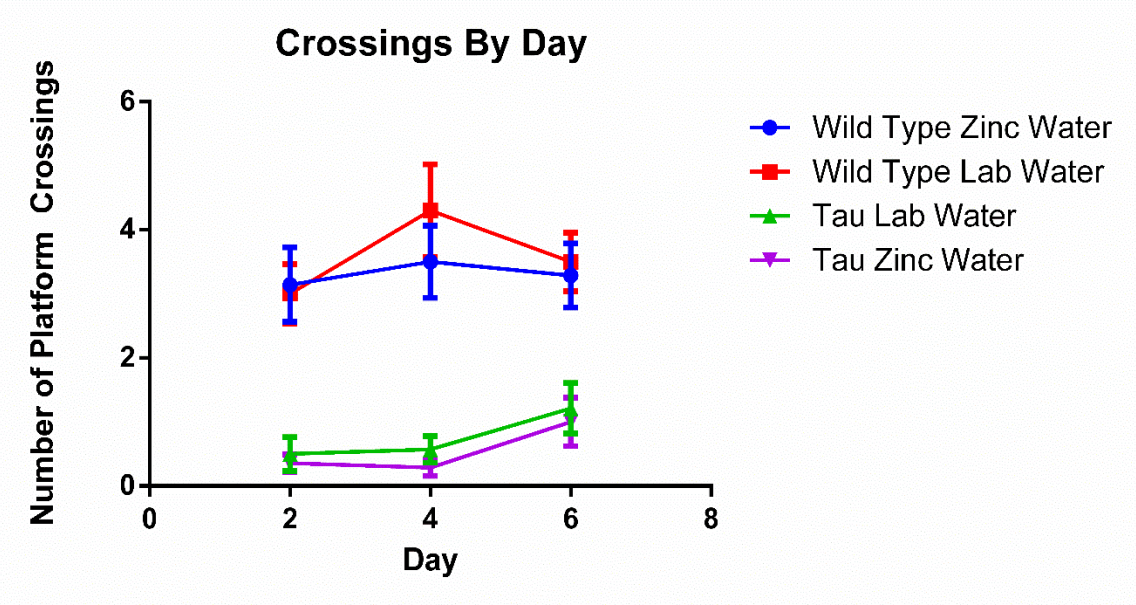

Figure 3- Number of platform crossings during hidden platform trials. Tau mice made significantly less crossings of the platform than wild-type mice; no interaction effects were observed.

Circadian Rhythm – A 2x2x5 Mixed Effects ANOVA was run to assess the time to onset of activity for mice. A significant interaction effect was found between genotype and type of water consumed, $F(1, 52) = 4.147$, $p < .05$. Post-hoc testing for this interaction shows both wild type groups being significantly different from both Tau groups ($ps < .001$) as well as Tau mice drinking zinc water being significantly different from Tau mice drinking lab water ($p < .01$), with Tau mice consuming zinc water having the latest onset time. This effect is shown in Figure 4.

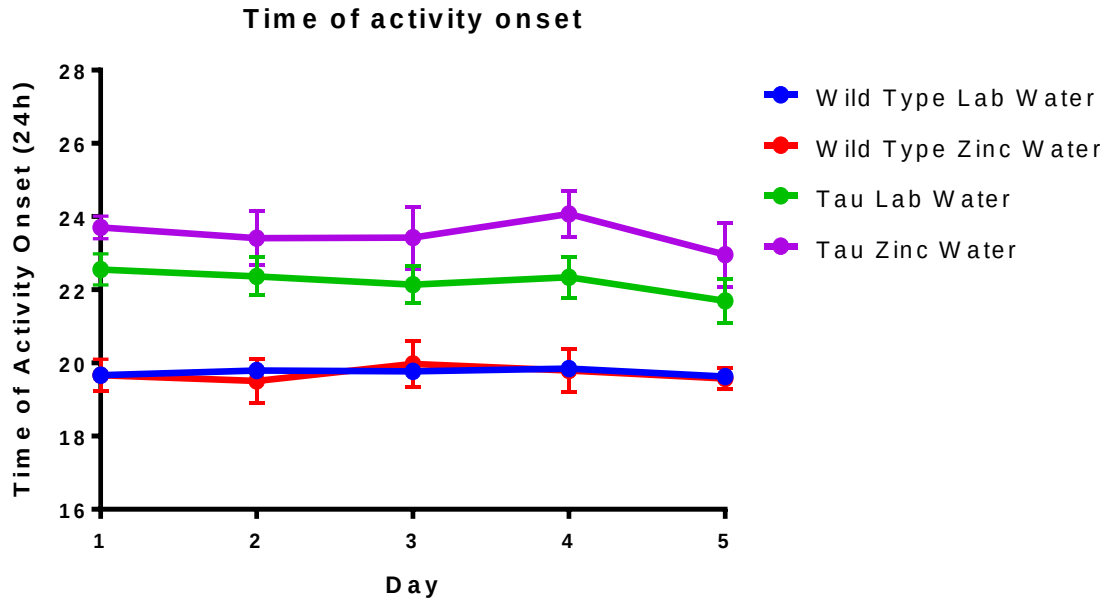

Figure 4- Time of activity onset as analyzed by wheel running activity. Wild type mice usually began running at just before 20:00 every day, which is just before lights come on in the colony. Tau mice began activity later than wild type mice with Tau mice who were consuming zinc starting later than Tau mice consuming lab water. There was a significant interaction effect for zinc and Tau for this measure.

Nesting- As seen in Figure 5, mice from different groups constructed visually different nests. Nests were scored on a scale of 1-5 by two blind observers. The scores were analyzed with a 2x2 ANOVA and a significant interaction effect of water and genotype was observed, $F(1, 52) = 6.021$, $p < .05$. Post-hoc testing for this interaction shows both wild type groups being significantly different from both Tau groups ($ps < .001$) as well as Tau mice drinking zinc water being significantly different from Tau mice drinking lab water ($p < .05$), with Tau mice consuming zinc water having the worst nests.

Figure 5- Left to Right: wild type lab water, wild type zinc water, Tau lab water, Tau zinc water. All cages started with bedding dispersed throughout the cage. These images were acquired two days after the mice were placed in these cages. Independent observers scored the nests and a significant interaction effect between zinc and Tau was found.

DISCUSSION

This project sought to examine the effect of low-level chronic zinc supplementation on mice containing the human Tau protein. Previous research both in vitro and in *Drosophila* models of learning has shown that zinc has the ability to exacerbate Tauopathy regardless of phosphorylation levels (Huang et al., 2014)(Kim et al., 2011). Elderly individuals are the most likely to have a Tauopathy and are also more likely to supplement their diet with additional zinc for a multitude of reasons including eye health and disease treatments. The Age-Related Eye Disease Study (AREDS) performed by the National Eye Institute specifically mentions benefits of supplementing with zinc. The behavioral tests conducted in this experiment were meant to mirror dysfunctions seen in Tauopathies including deficits in: spatial and short term memory, sleep-wake cycle, and daily living activities.

Morris Water Maze is a common test in animal research to assess short term and spatial memory. Previous research has demonstrated that many animal models of AD and Tauopathy have deficits in learning this task (Ma et al., 2014). In this study, wild type mice displayed a typical Morris Water Maze learning curve in both the lab and zinc water conditions. By day 4, the average time to find the platform was under 20 seconds for wild type mice. As expected based on previous research, Tau mice performed significantly worse than wild type mice, taking longer to find the platform on every day of testing (Ma

et al., 2014). Through the use of the correct combination of Tau genotype and promoter, we were able to successfully induce the expression of the Tau protein. This is an important step to note, as other researchers have neglected to find deficits in Tau mice because the protein was not expressed correctly. Zinc supplementation compounded these deficits causing there to be little to no learning shown at all. These results are strengthened when considering each cage used in this study was equipped with a running wheel for exercise, which has been shown to delay cognitive impairments.

The detrimental effect of zinc was not seen in two other Morris Water Maze measures: thigmotaxis and number of platform crossings. Thigmotaxis is a measure of the percentage of time the mouse spends along the outermost 10% of the maze. It has been used as a measure of anxiety in mice due to the exploratory nature of rodents, who are less likely to explore the center of an arena if they are anxious. There was a significant effect of genotype but not an interaction effect for this measure. Tau mice spending significantly more time than wild type mice around the outer edges of the pool may explain a portion of the results seen in assessing time to platform; however, post-hoc testing shows that zinc significantly exacerbated Tau mice in time to find the platform but did not show this same interaction effect for thigmotaxis. Thus, thigmotaxis cannot fully explain the results of time to platform. Open field testing on these same mice also did not result in an interaction effect for anxiety, which indicates that the specific effect of zinc on Tau may be more localized in the brain to cause deficits in memory but not in anxiety (Hernandez, SFN 2016). Histochemical analysis of the brains using immunohistochemistry showed zinc significantly exacerbated Tauopathic tangles in the

hippocampus but not in the prefrontal cortex or other areas of the brain (Craven, SFN 2016). This supports the idea that the effect of zinc supplementation in this model may be localized to certain portions of the brain. The heightened anxiety seen in the Tau mice may also partially explain the results seen in number of platform crossings due to Tau mice spending more time around the edges of the apparatus.

On days two, four, and six of MWM testing, the platform is lowered from its original location and made inaccessible to the mouse. For these trials the number of times the animal crosses where the platform would have been located can be used as a way to assess memory. Given the deficits seen in time to find the platform, it would be expected that the results for number of platform crossings would be similar. There was a main effect of genotype seen for number of platform crossings but there was not an interaction effect of genotype and zinc supplementation. The most likely reason for this is a floor effect; both transgenic groups of mice had on average less than one crossing. Additionally, because Tau mice spent significantly longer along the outside of the apparatus they were less likely to cross the platform on Atlantis trials. Because both Tau groups performed so poorly, there is no way a difference could be found between these groups.

The detrimental effects of zinc seen in the MWM were also seen in measures of daily living including sleep-wake cycle and nesting. Nest building in mice is an instinctual behavior that can be observed in all mouse colonies (Deacon, 2006). Pregnant female mice build nests out of the bedding in their cage before giving birth to a litter, and both male and female mice will assemble bedding into nests for shelter and comfort. A

major hallmark of Tauopathies including AD is a disruption in daily functioning for which nesting behavior in mice is a close parallel (Deacon, 2006). Wild type mice rearranged the scattered strips of paper to form a bed regardless of water condition. The majority of these nests included every single strip of paper although in some circumstances stray pieces of paper were not incorporated. Mice with the Tau gene that consumed lab water were able to build very rudimentary nests. Some pieces of paper were arranged together but the majority were not.

When the Tau mice were consuming zinc water this pathology was exacerbated to show no nest building and in most cases no re-arrangement of the paper at all. This significant interaction effect shows the same exacerbation of deficits seen in the MWM by the addition of zinc. This finding is particularly interesting as deficits in daily living are common in Tauopathies and these deficits combined with changes to sleep patterns are the most often reasons an elderly individual is placed into care facilities.

The mice used in this study are nocturnal animals who naturally are more active at night, to study their sleep-wake cycles we had to assess them both during the day and at night. This was done by placing the mice in individual cages with running wheels, which were attached to a computer that logged how often the wheel rotated. This method of assessing circadian rhythm has been used in research for a long time and was recently validated in a study using Tau mice that showed disruptions in wheel running activity are correlated with dysfunctions in the suprachiasmatic nucleus, which is responsible for setting circadian rhythms in mammals (Stevanovic et al., 2017). Naturally, mice usually

begin their activity just prior to nighttime and this natural rhythm is also seen in animals used in research environments.

Wild type mice usually began their activity within 20 minutes of the lights going out in the colony. This time of activity onset was not altered by the addition of zinc to wild type mice. Transgenic Tau mice have been noted to show difficulties in sleep-wake cycles and, on average, Tau mice consuming lab water had their daily activity begin 2 hours after wild type mice. The Tau mice consuming supplemental zinc had the start of their activity delayed an additional hour, starting wheel-running activity 3 hours after wild-type mice. The Tau deficits seen in sleep-wake cycle activity are similar to the deficits seen by patients suffering from diseases such as AD, and the addition of zinc compounded the already present deficits in the Tau mice.

The deficits found in this study combined with the histology performed on the mice points to a widespread and significant role of zinc in Tauopathies. As previously stated, patients suffering from Tauopathies have noted behavioral problems with spatial and working memory, sleep-wake cycle, and daily living activities. These same patients are more likely to be consuming zinc either in a multivitamin or at the recommendation of doctors as evidenced through AREDS. This study shows that the addition of chronic supplemental zinc to the transgenic mice exacerbated the natural deficits due to Tau in each of the behaviors tested. Histology performed post-mortem on these mice showed significantly higher levels of phosphorylated Tau and lower levels of free zinc in the brains of Tau mice consuming zinc water. This supports the hypothesis that the supplemental zinc is being taken in by Tauopathic neurons and disrupting them at an

accelerated rate. Mice in this study were first exposed to zinc at an age of 8 weeks old, which is important both because zinc has been known to cause developmental deficits in mice if given just after birth (Railey, Micheli, Wanschura, & Flinn, 2010) as well as to mimic the fact that elderly individuals are the most likely to supplement with excess zinc.

Future research on this topic could examine the potential therapeutic role of zinc chelation used concurrently with other treatments for Tauopathies. Additionally, the role of zinc in human subjects should be examined more closely. Zinc is necessary for normal functioning in humans and has been shown to help patients with eye problems; however, evidence shows excess zinc can also be detrimental, especially in patients with neurodegenerative disorders including Tauopathies and diseases that affect amyloid. Patients should be cautioned about the potential benefits and drawbacks to consuming supplemental zinc through multivitamins or other supplements.

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William Kochen graduated from Kellenberg High School in Uniondale in 2008. He completed his undergraduate education in Psychology and Biology with a Developmental Genetics concentration in 2013. While an undergraduate student, he completed research in comparative neuroanatomy using painted turtles as a model organism. As a PhD student at George Mason University, he has completed research in AD as well as designed a new model for Traumatic Brain Injuries in mice.