THE BIOCHEMICAL EFFECTS OF TAU AND ZINC IN A TAU MOUSE MODEL

by

Kristen Marie Craven
A Thesis
Submitted to the
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of
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in Partial Fulfillment of
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of
Master of Arts
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Committee:

_____ Director

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George Mason University
Fairfax, VA

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Bachelor of Arts
Wilkes University, Pennsylvania, 2014

Director: Jane M. Flinn, Associate Professor
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Fairfax, VA

DEDICATION

This is dedicated to my parents, Sam and Marie Craven, who have believed in me throughout every step of my education and provided their unconditional love and support.

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I would like to thank my committee, Dr. Jane M. Flinn, Dr. Geraldine Grant, and Dr. Tyler Shaw. The support and guidance on this project is greatly appreciated.

I would also like to thank my collaborators, William Kochen and Carlos Hernandez. This project has been a long time coming.

Lastly, thank you to my friends and family who stuck by my side while I worked on this project. Mom and Dad, you are always my biggest supporters. To my lab mates, you listened to my endless rants about my westerns not going as planned and provided me with invaluable feedback. Tauseef, you were always there to listen to the ups and downs.

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LIST OF ABBREVIATIONS

Alzheimer's Disease ..... AD
Microtubule Associated Protein ..... MAP
Neurofibrillary Tangles ..... NFTs

ABSTRACT

THE BIOCHEMICAL EFFECTS OF TAU AND ZINC IN A TAU MOUSE MODEL

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George Mason University, 2018

Thesis Director: Dr. Jane M. Flinn

Alzheimer's disease is a neurodegenerative disease affecting millions worldwide. The hallmark signs are progressive memory loss, confusion, lack of spatial awareness, and changes in personality. This debilitating disease has no known cure and many drug treatments have failed the final stages of testing. Hyperphosphorylated tau is a key pathology within the Alzheimer's disease brain as well as frontotemporal dementia, traumatic brain injury, and Parkinson's disease. Using a transgenic mouse model that contains the human gene for tau protein (P301L), this study assessed the effects of excess chronic zinc supplementation on tau pathology. Zinc is an essential trace element that is vital for neuronal communication and can be found in high concentrations in the hippocampus, a structure that is affected in Alzheimer's disease. Zinc has been shown to bind directly to tau and has also been shown to alter levels of phosphorylation. The pSer396 and Tau-5 antibodies were utilized in this study. The amount of phosphorylated tau protein at that site as well as the total amount of tau was semi-quantified (n = 4).

Furthermore, Zinpyr-1 was used to semi-quantify the amount of free-zinc (n = 4). Finally, Thioflavin-S was utilized to determine the presence of tau tangles (n = 3). Results show that transgenic tau mice supplemented with zinc water demonstrated higher concentrations of phosphorylation at Ser396 whereas total tau levels remained steady. Furthermore, transgenic tau mice had significantly less free-zinc in the hippocampus compared to wildtype mice as determined by Zinpyr-1 staining, indicating that tau tangles may be sequestering the zinc. Finally, transgenic tau mice given zinc water demonstrated a higher number of tangles in the hippocampus at 7 months of age when compared with transgenic tau mice given standard lab water. These results show that zinc exacerbates tau pathology. Caution should be taken with regard to zinc supplementation, particularly in populations that are already at-risk of tauopathies such as Alzheimer's disease.

CHAPTER ONE: INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that affects millions worldwide and the number of individuals suffering is expected to increase substantially. It is the most common form of dementia and while research has sought to determine the underlying mechanisms they are still unknown. It is estimated that approximately five million individuals suffer with AD in the United States alone. This number is only expected to increase, as people are living longer and no cure or promising treatment has been found. Many drugs have failed to pass the final stages of testing, leaving those with the disease as well as their caregivers with little hope. AD starts as a slow decline in short term memory. As the disease progresses changes in personality occur and eventually a complete loss of short term and spatial memory are evident. There are two different forms of AD: early and late onset, with late onset occurring after the age of 65 and being the most common. Aside from the behavioral symptoms, this disease is also characterized by amyloid beta plaques which are found outside of neurons as well as hyperphosphorylated tau tangles, which are found inside neurons. It is thought that these two features are what lead to the loss of neuronal connections and therefore declines in short-term and spatial memory (Alzheimer's Association).

This research focuses on tau pathology. In addition, the effect of zinc on tau will be assessed, which will be discussed in detail later. Tau is a microtubule associated

protein (MAP) which normally binds to and stabilizes microtubules through the interaction with tubulin (Weingarten, Lockwood, Hwo, & Kirschner, 1975). Tau is a phosphoprotein, meaning it is normally in a phosphorylated state which is dependent on kinase and phosphatase activity. The main kinases responsible for phosphorylation of tau include PKA, PKC, and CaMPK II. These kinases phosphorylate at different sites on tau with varying overlap (Lovestone & Reynolds, 1997). Furthermore, tau goes through other post-translational modifications such as glycosylation and ubiquitination, which may also affect phosphorylation (Wang et al., 2013). The phosphorylation of tau has been shown to be vital to its ability to bind microtubules (Lindwall & Cole, 1984; Selden & Pollard, 1983).

However, in the instance of AD tau becomes hyperphosphorylated and is no longer able to carry out its normal function (Iqbal et al., 1986). This leads to an inability to bind to microtubules, causing breakdown and dysfunction. Chemical transport becomes affected and tau protein slowly builds up because it no longer has an affinity to bind to tubulin. These tau proteins progress to form paired helical filaments (PHF), resulting in neurofibrillary tangles (NFTs), a hallmark of AD (Iqbal et al., 1986). In addition, normal tau has the affinity to bind to tau in its hyperphosphorylated state (Alonso, Iqbal, & Iqbal, 1996).

Hyperphosphorylation occurs at many different serine/threonine sites (See Figure 1). One of these sites, Ser396 has been found to be hyperphosphorylated in the earlier stages of the disease (Rodriguez et al., 2014) and its importance for disease progression has been illustrated (Bramblett et al., 1993). On the other hand, AT8, an antibody specific

for phosphorylation at Ser199, 202, and Thr205 has been frequently used in research (Augustinack, Schneider, Mandelkow, & Hyman, 2002; Dumont et al., 2011; Rodriguez et al., 2014) and it has been determined that this antibody recognizes tau pathology in the later stages of AD. It is not clear why phosphorylation takes place at specific sites earlier than others; however it is clear that there is a specific cascade that must take place with regards to the progression of AD. Using eleven different tau antibodies, Augustinack and colleagues (2002) showed the progression of sites of hyperphosphorylation in AD. It was discovered that some antibodies recognize only pre-NFTs while others recognize intracellular NFTs. The antibody for Ser396 recognizes both intracellular and extracellular NFTs.

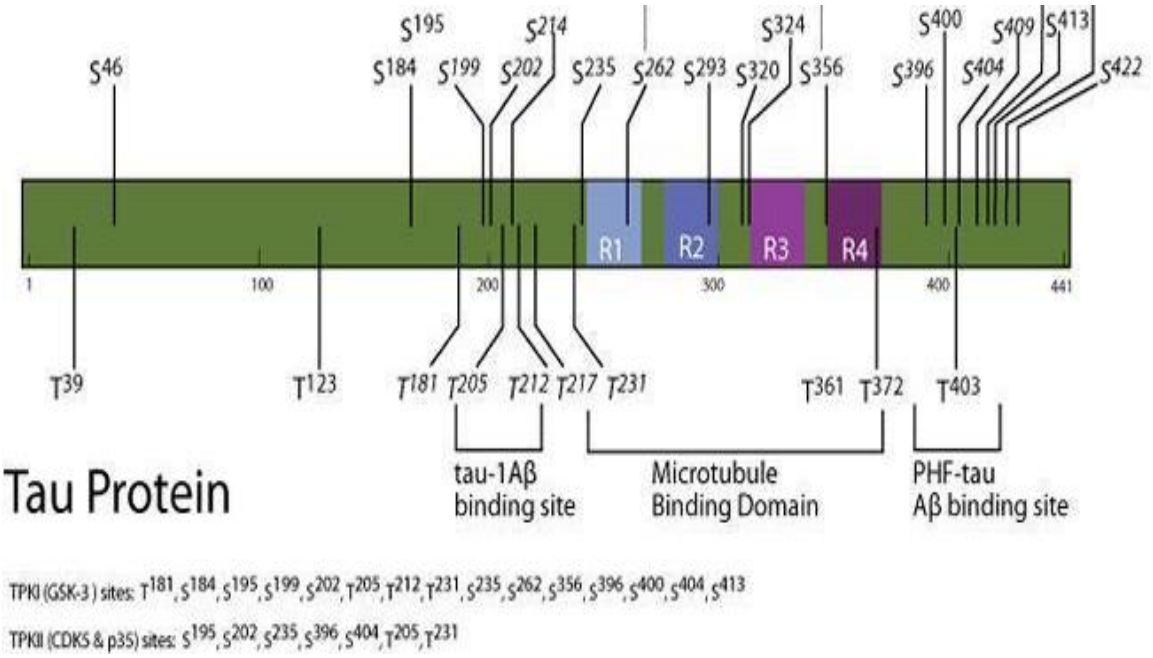

Figure 1. Various Phosphorylation Sites on Tau Protein. This research focused on phosphorylation at Ser396, the PHF site. The microtubule binding domain is where changes are made in order to generate different isoforms of tau protein (Retrieved from GeneTex.com, Tau Antibody, 2017).

With regards to Augustinack's research it is important to clarify the distinction between pre, intracellular, and extracellular NFTs. Pre-NFTs (pNFTs) are found in the earlier stages of AD. Augustinack classified these neurons as having an intact nucleus and dendrites, however small tau deposits can be observed in the cell body of the neuron. These were primarily found in the entorhinal cortex, one of the first areas affected by AD. Intracellular-NFTs (iNFTs) were considered to have an intact nucleus, albeit seemingly out of place, as well as damaged dendrites. In extracellular-NFTs (eNFTs), no nucleus or dendrites were visible. The presence of eNFTs is indicative of a more advanced stage of AD (Augustinack, Schneider, Mandelkow, & Hyman, 2002). Of interest, Augustinack used PHF-1, which primarily recognizes eNFTs as well as iNFTs at Ser396 and Ser404 (See Figure 2). The site-specific antibody for Ser396 will be utilized in the current study.

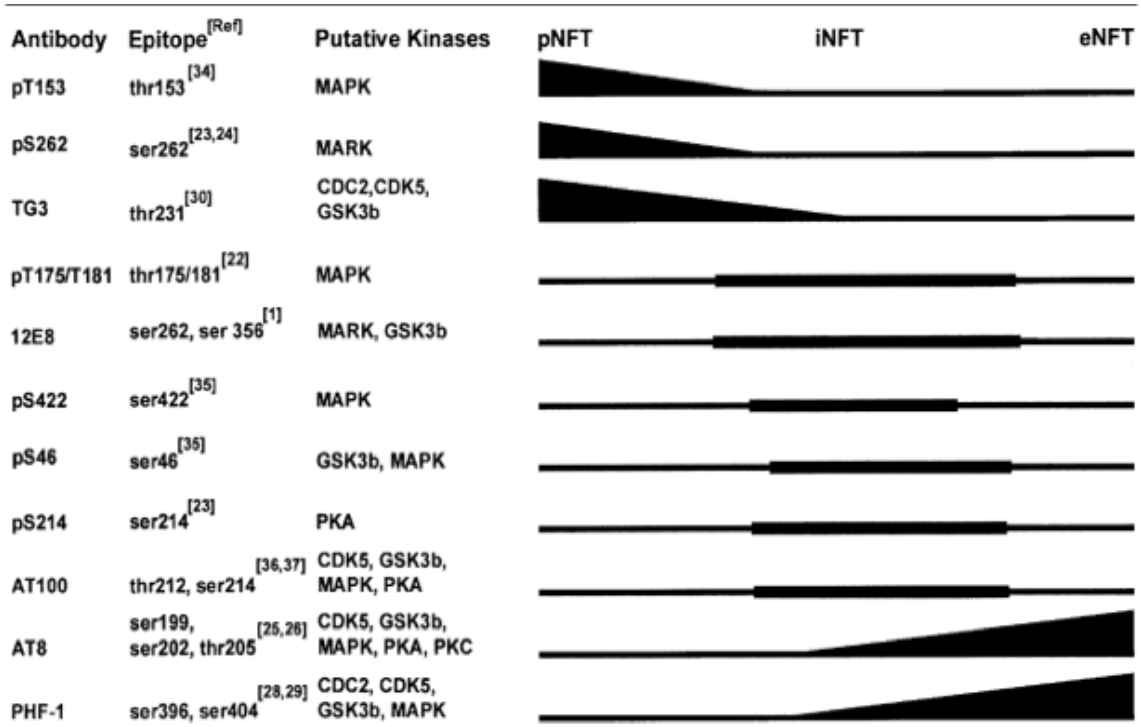

Figure 2. Common Tau Antibody Labeling. The Ser396 site is phosphorylated early on in AD, and is also indicative of advanced pathology, as intracellular and extracellular tangles are typically seen at this site (Augustinack et al., 2002).

A study conducted by Rodriguez et al (2014) found that phosphorylation at Ser396 occurs earlier than phosphorylation at the AT8 epitope. However, another study completed by Dumont et al (2011) used AT8 in combination with behavioral testing in the P301S transgenic mouse model. This model is similar to the P301L model that was used in the current study. Both animal models develop tau tangles and can be used to study that pathology in AD. Dumont and colleagues found that number of NFTs at the AT8 epitope increased between 2 and 10 months. Behavioral deficits were seen by 7 months on the elevated plus maze; however, no differences were seen when compared to wildtype mice on the Morris Water Maze. Their results ultimately showed that behavioral deficits and oxidative stress occur before tau pathology in AD. Aside from the Dumont

(2011) study, Kanno and colleagues (2014) used the 5XFAD transgenic mouse model and found contradicting results. Their research showed that the Ser396 site was hyperphosphorylated earlier than the appearance of behavioral deficits rather than later. The 5XFAD mouse model is an early onset AD model. These mice develop intense amyloid deposition, whereas tangles are not often seen, as the gene for human tau is not present. The discrepancy between the above two studies can be easily explained by the fact that the 5XFAD mouse does not model human tau pathology. The current study assesses the number of NFTs as well as the amount of pSer396 at 7 months of age in a tau mouse model, allowing the focus to be placed on the specific role of human tau in disease progression.

NFTs can be assessed using Thioflavin-S staining. Previous studies have determined that Thioflavin binds to paired-helical filaments, which are what tangles are primarily made up of (Alonso, Iqbal, & Iqbal, 1996). Gotz and colleagues (2000) utilized Thioflavin-S and determined that NFTs were present in the P301L tau mouse as early as 3 months of age. Another more recent study (Cook et al., 2015) also utilized Thioflavin-S in the P301L mouse and found NFTs at 6 months of age. However, Lewis and colleagues (2000) only found pre-tangles in the hippocampi of these mice at 4.5 months of age. The distribution of when and where NFTs appear in these mice is not consistent. The Thioflavin-S method has been previously improved (Sun, Nguyen, & Bing, 2000) to allow for better visibility of NFTs. Thus, it is also possible that varying protocols may lead to different results at the different ages. This study aimed to determine the presence of NFTs at 7 months of age in the hippocampus using the modified Thioflavin-S method.

As previously stated, this research does not only focus on the role that tau plays in AD. Increasing evidence supports the interaction of tau with the essential trace element zinc. It has been shown that zinc can affect NFTs (Huang et al., 2014; Sun et al., 2012; Zhou, Chen, & Xiong, 2012). Zinc plays structural and functional roles, as well as affects the regulation of specific proteins (Nuttall & Oteiza, 2014). Furthermore, zinc is important for memory, transmission between neurons, differentiation of neurons, and is co-released with neurotransmitters such as glutamate (Maret & Sandstead, 2006). It is found in high concentrations in the hippocampus, amygdala, and striatum, as well as the parietal lobe in AD patients (Frederickson et al., 1987; Shrag et al., 2011).

There are different forms of zinc within the body. These include circulating zinc (free-zinc), protein-bound zinc, and zinc contained in vesicles (with glutamate) (Frederickson et al., 2000; Hancock, Finkelstein, & Adlard, 2014; Nuttall & Oteiza, 2014). Protein-bound zinc is the most abundant form of zinc within the brain (Frederickson, 1987). It is unknown how each of these specifically affects NFT development in AD; however research has shown that free zinc is found in significantly lower concentrations in individuals with AD (Brewer, 2012; Pepersack et al., 2001; Prasad et al., 1993). Free zinc concentrations in the brain have been measured through the use of Zinpyr-1 (Frederickson et al., 2004; Woodroofe, Masalha, Barnes, Frederickson, & Lippard, 2004). This is a useful technique to visualize the distribution of zinc, as fluorescence is used. It is known that high concentrations of zinc can be found in the AD hippocampus and as such was looked at in this research (Deibel, Ehmann, & Markesbery, 1996).

While the specific interaction of zinc and tau has not been determined, Akiyama and colleagues (2012) attempted to clarify this link. Although they did not focus on a tau only mice model, their results illustrated that increasing zinc in the animals' drinking water does not increase the development of plaques and tangles. However, no behavioral testing was carried out and previous research has illustrated that increased zinc has a negative impact on cognition (Flinn et al., 2005; Railey, Micheli, Wanschura, & Flinn, 2010). In addition, Bush et al,. (1994) determined that amyloid beta can bind zinc. These studies taken together indicate that levels of zinc are not the only modulators of AD pathology but that they do play a key role in the effects on cognition.

Increased levels of zinc have been shown to contribute to the hyperphosphorylation of tau, which ultimately leads to the development of NFTs (Nuttall & Oteiza, 2014, Sun et al., 2012). Huang and colleagues (2014) experimented with drosophila and found that zinc increases tau tangle levels not only by increasing the phosphorylation of tau, but also by directly binding to tau. They determined that both mechanisms play an important role in the development of NFTs. The specific mechanism of how zinc increases the phosphorylation of tau has not been fully determined, however it was discovered in human blastoma cells that this may occur through the upregulation of kinases. Specifically, this was at the Ser262/356 site and hydrogen peroxide was shown to decrease this phosphorylation (Zhou, Chen, & Xiong, 2012). Taken together with the Huang study, there is still much to determine about the specific effects of zinc on tau; for instance, it is not known why phosphorylation at that site is seen, or if findings from blastoma cells and drosophilia can be taken together and generalized to the population.

Furthermore, while Zhou and colleagues' research focused on one specific epitope, there are many more sites on tau that have not been explored and it is not known whether there is a link between those sites and zinc.

While *in vitro* work has shown that zinc can bind directly to tangles as well as increase levels of phosphorylation, no previous studies have observed the effects of zinc on tau *in vivo*. This is important because zinc is found in many dietary supplements, insulin sprays, and has been prescribed for macular degeneration. Because of the role that zinc plays in everyday life, it is vital to study the effects on tau, as an already at-risk elderly population are likely to be supplementing their diet, using insulin sprays, and being prescribed medications for macular degeneration. A major concern is that the role of zinc in the brain and in the progression of AD is not fully understood. This study sought to determine whether zinc affects behavior and biochemical pathology *in vivo*.

Hypotheses

The current study assesses the role of tau tangles and excess zinc supplementation by utilizing biochemical methods. This will be accomplished using a transgenic tau mouse model of AD, generated by breeding the P301L mouse and CamKII promoter mouse from the Jackson Laboratory. This mouse phenotypically expresses behavioral deficits associated with AD such as decreased spatial memory and short term memory as well as the presence of tau tangles, assessed biochemically. More specifically, the P301L mouse only has the genotype for tau. The CamKII promoter mouse allows this gene to be expressed phenotypically and allows us to assess the effects of AD in this model through

behavioral testing. Results from this AD model will be compared with a Wildtype mouse model. This research is important with regards to AD because this mouse model had not previously been tested when raised on water with excess zinc. As discussed, zinc is thought to exacerbate the effects of AD. Four experimental groups were tested: Wildtype mice on lab water, Wildtype mice on zinc water, P301L/CamKII (tau) mice on lab water, tau mice on zinc water. The animals on lab water are control groups and the animals on zinc water are experimental groups. The hypotheses are as follows:

1. There will be an increase in pSer396 levels between wildtype and transgenic mice as assessed through the use of Western Blotting.

a. Wildtype mice are expected to show very low levels of pSer396, as they do not have the human gene for tau.

b. Transgenic mice on lab water and zinc water are expected to show increased levels of pSer396 with the latter demonstrating higher levels. This is expected due to the fact that it is hypothesized that those with excess zinc supplementation show exacerbated AD pathology.

2. There will be a difference in reactivity to Zinpyr-1, a probe for free-zinc, between wildtype and transgenic mice in the hippocampus.

a. Wildtype mice given lab water will show little reactivity to Zinpyr-1 as compared with wildtype mice given zinc water.

b. Transgenic mice given lab water will show less reactivity to Zinpyr-1 when compared with wildtype mice given zinc or lab water.

c. Transgenic mice given zinc water will show the lowest reactivity to Zinpyr-1 when compared with all other groups. Transgenic mice are expected to show lower reactivity than wildtype mice because zinc directly binds to tau. This would lower the amount of free-zinc in these mice.

3. There will be a difference in the number of tau tangles as determined by Thioflavin-S staining such that transgenic mice given zinc water would have the highest number of tangles.

a. Wildtype groups will show no tau tangles.

b. Transgenic tau mice given zinc water will have a higher number of tangles in the prefrontal cortex and hippocampus compared to transgenic tau mice given lab water.

CHAPTER TWO: MATERIALS AND METHODS

Animal Models and Breeding

A total of 58 mice were used in this experiment for behavioral testing. This included 29 transgenic P301L/CamKII mice and 30 Wildtype mice. To reiterate the importance of the CamKII promoter mouse, this allowed the phenotype of impaired short term and spatial memory as well as the presence of tau tangles to be seen. Fifteen of the transgenic mice were given excess dietary zinc in the form of 10ppm zinc water while the other 14 were given lab water. Wildtype mice were broken down into groups of 15 on zinc water and 15 on lab water.

For breeding, P301L females were separated into three cages of three females each and 3 CamKII males were separately housed together for two weeks. Love mash (BioServ) supplementary diet was given to the mice two weeks before pairing. This diet contains extra fat to prepare the animals for breeding. This is especially important for females since proper nutrition is so vital to being able to carry and give birth to a litter. The males were separated into individual cages after the two-week acclimation period in order to prepare for pairing with the females. Bedding was transferred from the male cage into the female's cage that the particular male was set to be paired with. This occurred five days before pairing. Males and females were paired for two weeks. The females were then placed into individual cages and the males were placed back into the same cage as

their original cage mates. Litters were born approximately 21 days after the first day of pairing.

Offspring were genotyped by cutting approximately 4mm of the end of the tail and sending this to Transnetyx, a company specializing in genotyping. Between 11 and 21 days this procedure is relatively painless to the pups as they do not have sensation at the end of their tails. Once the results from Transnetyx were obtained animals were weaned into cages of approximately 4 males and 6 females, respectively. Eight weeks after birth the mice in the zinc water groups were started on this condition. This allowed for proper brain development to occur before administering an excess in dietary zinc.

Zinc water was prepared using a 10ppm solution of Zn dissolved in 5% nitric acid. Sodium carbonate (NaCO3) was added 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 for metal content using inductively coupled plasmaoptical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS, Reston,VA). All mice were given food (7012 lab diet, Harlen Laboratory) and water *ad libitum*. All animals continued on their separate water conditions until the end of the study, at approximately 7 months after birth, at which point animals were sacrificed using gradual CO2 asphyxiation and subsequent decapitation. At this point brains were extracted for tissue analysis and immediately placed on dry ice. Brains were stored in a -80°C freezer until analyses.

Western Blot

Brain tissue was extracted and immediately frozen on dry ice. Samples were stored in a -80℃ freezer until homogenized. The right hemisphere (n = 4 per condition) was placed in 1mL of RIPA buffer on ice with Halt Protease and Phosphatase Inhibitor Cocktail (ThermoScientific) at the recommended concentration of 1µL/mL. Samples were homogenized and subsequently centrifuged at 14,000RPM for twenty minutes at 4℃ and aliquoted. The BCA assay was run to determine protein concentrations. Sample absorbances and protein concentrations were read using a spectrophotometer. Samples were prepared using 40µg of protein, 2.5µL NuPAGE sample reducing agent, 6.25µL LDS sample buffer, and PBS for a final concentration of 25µL. Samples were placed in a 37℃ water bath for thirty minutes and loaded into NuPAGE 4-12% Bis-Tris gels in MOPS running buffer. SeeBlue Plus2 protein ladder was used to visualize molecular weight (ThermoScientific). The gel was run at 120V for approximately two hours and then transferred using the iBlot 2 Transfer System with mini nitrocellulose transfer stacks (Novex).

The membrane was washed with PBST for 3 minutes and then blocked in 5% milk for 30 minutes with agitation. Membranes were incubated with primary antibodies at 4℃ in 2.5% milk block. Primary antibodies included pSer396 (1:10,000; Abcam) and Tau-5 (1:1000; ThermoScientific). Cytochrome-C (1:1000; Bioss) was used as a loading control. After primary antibody incubation, membranes were washed with PBST 3 times for 10 minutes each and then placed in 2.5% milk block for 30 minutes. Membranes were then incubated with HRP conjugated secondary antibody (1:5000 Donkey anti-rabbit and

1:2000 Goat anti-mouse, respectively; ThermoScientific) and washed with PBST 3 times for 10 minutes each. West pico chemiluminescent substrate (SuperSignal) was used for 6 minutes and blots were subsequently imaged with an exposure time of 10 seconds. Images were semi-quantified using ImageJ (NIH).

Zinpyr-1

Fresh frozen brain tissue (stored at -80℃, *n* = 4 per condition) was sliced at 16μm using a Leica CM3050S cryostat. A 1mM stock solution of Zinpyr-1 (ThermoScientific; excitation/emission: 490/530) in DMSO was made, from which a 17μM working solution was made using .9% saline. Slides containing hippocampal slices were immediately incubated with enough working solution to cover each individual slice for 2.5 minutes. The solution was poured off and images were immediately imaged using an Olympus BX51 fluorescence microscope with a FITC cube. Magnification was set to 2x, green levels were set to 1.26, and exposure time was 1/1.8 seconds for all images. ImageJ software (NIH) was used to semi-quantify the images by first subtracting the background and subsequently using five different regions of interest in both the dentate gyrus and CA3 of the hippocampus to obtain fluorescent values for the amount of free-zinc.

Thioflavin-S

Thioflavin-S staining was used to determine the presence of tau tangles. Fresh frozen brain tissue (stored at -80℃, *n* = 3 per condition) was sliced at 16μm using a Leica CM3050S cryostat. Hippocampal and frontal cortex sections were mounted onto slides and post-fixed using a 4% PFA solution for 8 minutes. Slides were then washed with PBS and placed into a .25% potassium permanganate solution for 4 minutes followed by a 1%

sodium borohydride solution for 4 minutes as per a modified Thioflavin-S method (Sun et al., 2003). Slides were washed with distilled water and then placed into .05% Thioflavin-S in 50% ethanol for 8 minutes. Differentiation in two washes of 80% ethanol was performed followed by 3 washes of distilled water. Finally, slides were incubated with 10x PBS for 30 minutes at 4℃ and imaged using a FITC cube (as described above). Magnification was set to 40x, green levels were set to 1.26, and exposure time was 1 second for all images. ImageJ software (NIH) was used to overlay images in order to intensify the tau tangles for visualization purposes. Tangles were subsequently counted.

Design

A 2X2 ANOVA was used for western blot analyses, a 2X2X2 mixed factorial ANOVA was used for Zinpyr-1 staining, and a 2x2 mixed factorial ANOVA was used for Thioflavin-S staining. For western blot the two independent variables were genotype (wildtype, transgenic) and water type (lab, zinc). The dependent variable for western blot was relative densities calculated through the use of ImageJ (NIH). Independent variables for Zinpyr-1 included genotype, water type, and area (dentate gyrus and CA3 of the hippocampus) and the dependent variable was average green fluorescent values. The independent variables for Thioflavin-S staining were water type and area (prefrontal, hippocampus). Wildtype mice were not included in this aspect of the analyses, as it was evident that no tau tangles were present in samples. The dependent variables for Thioflavin-S staining were the number of tau tangles. It was expected that there would be an interaction between genotype and water type for western blot and Zinpyr-1 analyses such that zinc water would exacerbate tau pathology. It was also expected that tau mice

given zinc water would have a significantly higher number of tangles in the hippocampus. If no interaction was found significant main effects were followed up with main comparisons. If a significant interaction was found it was followed with simple effects. All analyses were subject to Levene's test of homogeneity.

CHAPTER THREE: RESULTS

Phosphorylation at Ser396 and Total Tau Levels

Western blots were completed using Tau-5 (total tau) and pSer396 antibodies (Figure 1). A 2x2 ANOVA showed tau mice demonstrated higher expression of phosphorylation at Ser396 compared to wildtype mice (F (1, 12) = 237.287, p = 0.000, partial eta^2 = 0.952). Furthermore, there was a significant interaction of genotype and water type for pSer396 (F (1, 12) = 15.603, p = 0.002, partial eta^2 = 0.565). Simple effects showed tau mice given water supplemented with zinc showed significantly higher expression of phosphorylation levels at Ser396 compared to tau mice given lab water (p = 0.000). Tau mice exhibited significantly higher expression of total tau compared to wildtype mice (F (3, 12) = 112.691, p = 0.000, partial eta^2 = 0.904). Levels of total tau did not differ between zinc and lab water tau mice (F (1, 12) = 0.461, p = 0.510, partial eta^2 = 0.021).

Zinpyr-1

Zinpyr-1 fluorescent stain was used to assess the difference in free-zinc concentration in the hippocampus (Figure 2). A 2x2x2 mixed effects ANOVA revealed a significant main effect of genotype. Transgenic tau mice had significantly less free-zinc in the hippocampus when compared with wildtype mice (F (1, 12) = 25.598, p = 0.000, partial eta^2 = 0.681). Overall, there was a significant within-subjects effect such that free-zinc levels were significantly higher within the dentate gyrus when compared with CA3

for all groups (F (1, 12) = 142.893, p = 0.000, partial eta^2 = 0.923), as expected. All other effects were nonsignificant.

Thioflavin-S

In order to determine the presence of tau tangles, Thioflavin-S staining was used (Figure 3). A 2x2 mixed factorial ANOVA revealed a significant main effect of water (F (1, 4) = 8.627, p = 0.043, partial eta^2 = 0.683), such that tau mice given zinc water had significantly more tangles in the hippocampus when compared with tau mice given lab water ($\bar{x}$ = 23, SD = 6.25, tau zinc; $\bar{x}$ = 3.33, SD = 1.53, tau lab). There was no significant difference between tau mice given lab or zinc water for the number of tangles in the prefrontal cortex ($\bar{x}$ = 21, SD = 8.19, tau zinc; $\bar{x}$ = 17.33, SD = 8.08, tau lab).

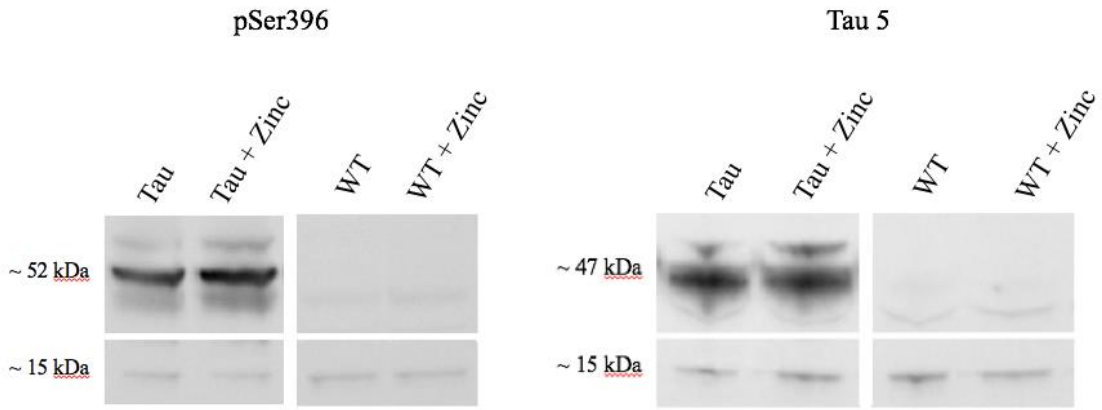

Figure 3. Western Blot Analyses. Tau zinc mice were found to demonstrate significantly higher levels of expression of phosphorylated tau at Ser396 compared to tau lab mice. Expression of total tau was not found to be significantly different between the two groups. Tau mice demonstrated significantly higher levels of total tau and pSer396 compared to wildtype mice overall. Bands at ~15kD show loading controls.

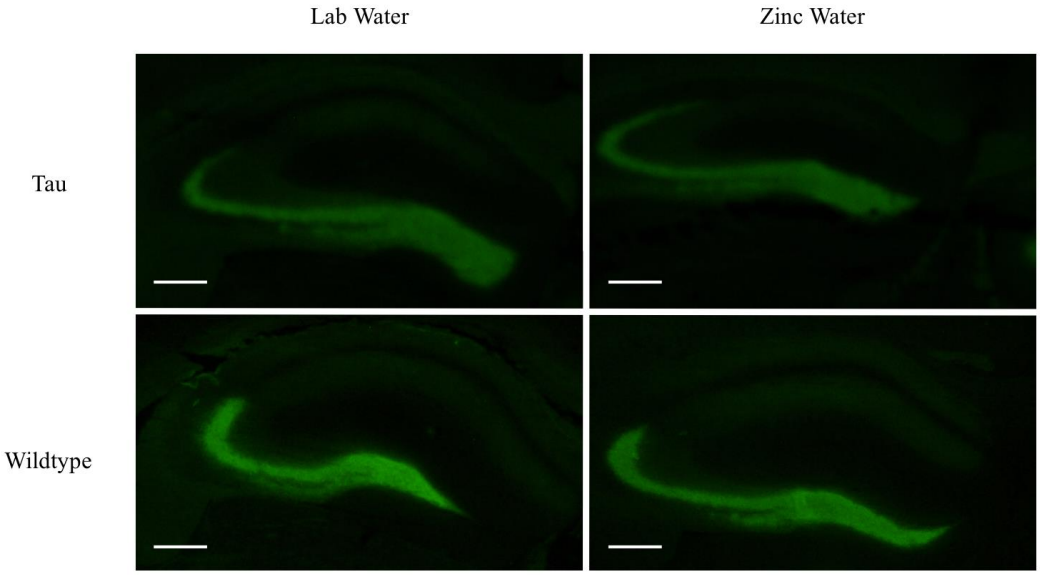

Figure 4. Zinpyr-1 Staining. Zinpyr-1 results demonstrate that tau mice have significantly less free-zinc in the hippocampus compared to wildtype mice. Scale bars represent 250μm.

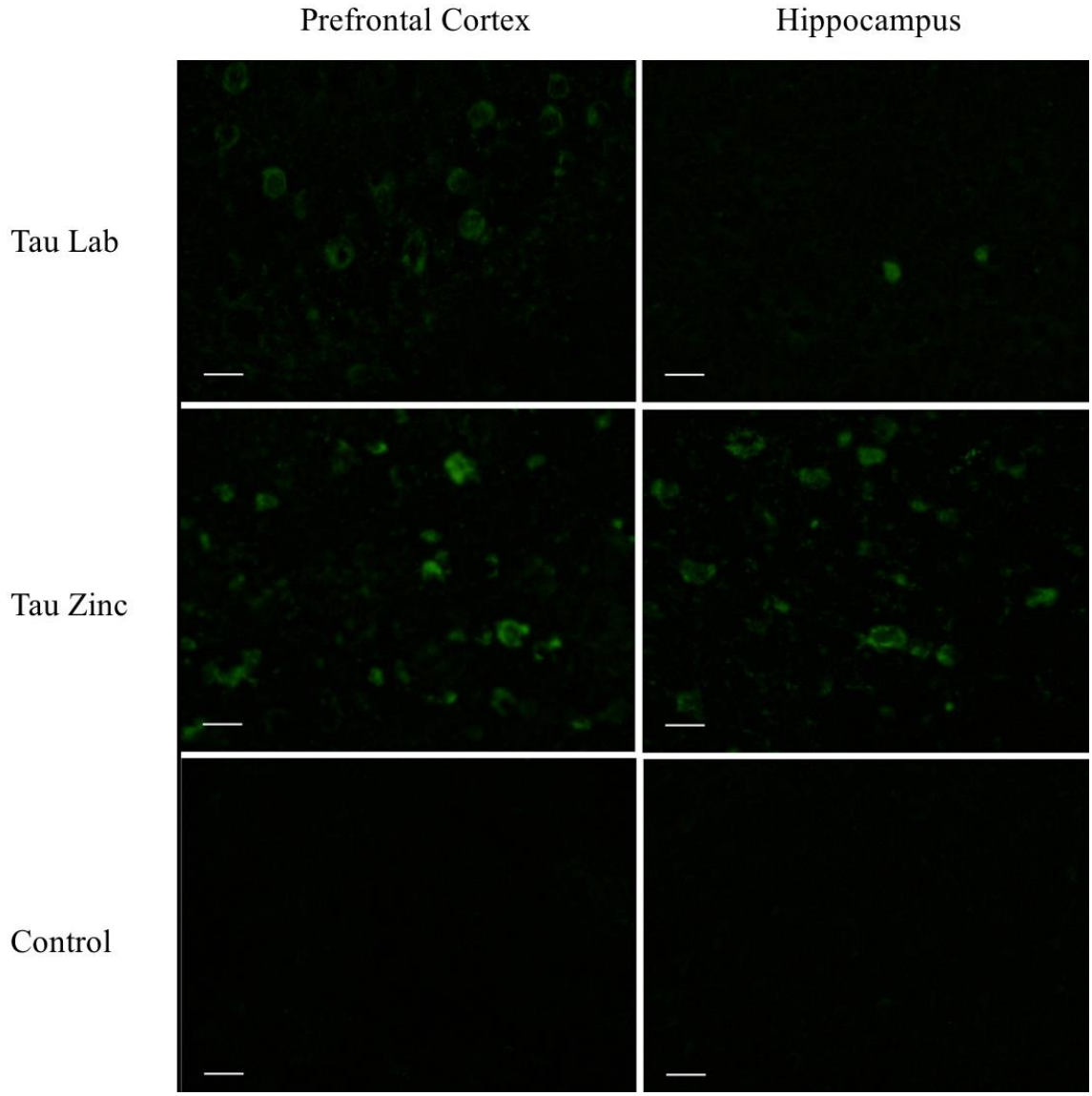

Figure 5. Thioflavin-S Staining. Tau zinc mice have significantly more tangles in the hippocampus compared to tau lab mice (23 and 3.33 on average, respectively). Tangles in the prefrontal cortex of tau zinc mice appear to show more intense fluorescence in the frontal cortex compared to tau lab mice, however tangle number did not significantly differ (21 and 17.33 on average, respectively). Scale bars represent 500μm.

CHAPTER FOUR: DISCUSSION

Through western blot and fluorescent staining, this research determined that excess zinc leads to exacerbated tau pathology progression. The main findings were as follows: western blot revealed similar amounts of total tau for tau mice given lab water and tau mice given zinc water with higher levels of phosphorylated tau at Ser396 for the tau mice given zinc water, Zinpyr-1 staining revealed higher concentrations of free zinc in the hippocampus in wildtype mice compared with transgenic tau mice, and Thioflavin-S tau tangle staining revealed a higher number of tangles in the hippocampus of tau mice given zinc water when compared with tau mice given lab water.

Western blot results showed that excess zinc leads to exacerbated tau pathology progression such that phosphorylation at Ser396 was increased. This expands on current literature showing that zinc directly binds to tau (Huang et al., 2014), demonstrating that not only can hyperphosphorylation at this site be linked to behavioral deficits, but that zinc exacerbates these deficits. It should be noted that there are many sites on tau that are impacted by hyperphosphorylation in Tauopathies and AD, only one of which was studied in the current experiment. However, previous work (Augustinack et al., 2002) has demonstrated that the Ser396 epitope correlates with intense NFTs, found in the later stages of AD. As such, it can be inferred that these 7 month old mice have progressed to an advanced state of tauopathy.

Similar levels of total tau by way of western blot, which was expected, indicate that the increase in expression of phosphorylation at Ser396 is indeed due to the contribution of zinc rather than variable levels of total amounts of tau. Dumont and colleagues (2011) also found similar total tau levels, as this antibody only recognizes the sheer amount of tau rather than taking into consideration the amount of phosphorylation. Tau protein is normally phosphorylated however, tau tangles begin to develop when hyperphosphorylation and dysfunction of the protein begins to take place. The increase in expression at Ser396 indicates a faster disease progression and showcases the detrimental effects of excess zinc on tau protein.

Because tau sequesters zinc (Huang et al., 2014), our water effect for Zinpyr-1 may have been lost, as both tau lab and tau zinc groups had tangles throughout the frontal cortex and within the hippocampus. Ongoing research within our lab has indicated that mice supplemented with zinc water have higher free zinc levels in the hippocampus. Further research on the effect of tangles sequestering free-zinc is needed.

Finally, Thioflavin-S staining for tau tangles showed that tau mice given zinc water had a significantly higher number of tangles in the hippocampal area compared with tau mice given lab water. Number of tangles in the frontal cortex did not significantly differ, although tau mice given zinc water do appear to have tangles with more intense fluorescence, which may indicate a higher density and therefore exacerbated pathology. Tau tangles appear in a number of neurological disorders including Alzheimer's disease, Parkinson's disease, and Frontotemporal Dementia, however the affected areas and disease progression differ between the disorders. We used the P301L

tau mouse model in this experiment to determine the effect of zinc on tau protein in a broad sense, however it should be noted that this mouse develops tangles in the frontal cortex first followed by progression into the temporal cortex where the hippocampus is located. Alzheimer's disease patients demonstrate tau tangles starting in the entorhinal cortex of the hippocampus with progression into the surrounding cortices and eventually the frontal cortex (Braak & Braak, 1991; Liu et al., 2012). Future studies examining the specific role of tau in Alzheimer's disease should focus on a tau mouse model that develops tangles in the hippocampus followed by the surrounding cortical areas.

Previous research has shown that zinc negatively affects amyloid protein such that zinc is required for the uptake of amyloid beta into dentate granule cells and by blocking this uptake memory can be salvaged (Takeda et al., 2017). Furthermore, a previous study in our lab (Flinn et al., 2014) showed that zinc supplementation negatively affected the special memory of a transgenic mouse containing genes for ApoE4 and amyloid beta (CRND8/E4), once again showcasing the detrimental effects of zinc. The current work expands on these findings and now shows that both amyloid and tau, two key proteins involved in Alzheimer's disease, are negatively affected by the trace element zinc.

Another possibility as to why zinc exacerbates the effects of abnormal tau protein is the role that copper plays. Zinc supplementation has been shown to lead to a copper deficiency (Maret & Sandstead, 2006; Voss et al., 2014). Behavioral deficits observed due to zinc supplementation were remediated by either copper supplementation in the water in Sprague Dawley rats or suppression of oral zinc administration in tau mice (Railey et al., 2006; Railey et al., 2010; Voss et al., 2014). Furthermore, it has been found

that copper increases amyloid precursor protein production and tau pathology in neurons in the hippocampus (Kitazawa et al., 2009) in a double transgenic mouse model of AD. While we have shown here that zinc exacerbates tau deficits biochemically and previous research has demonstrated that zinc directly binds to tau (Huang et al., 2014), it would be of interest to further explore the role that copper plays. The balance of these two trace elements is clearly vital in tauopathies and AD.

While this manuscript focusses on the biochemical aspects of the study, behavioral testing assessing spatial memory, wheel-running behavior, nest building, open field, and fear conditioning was also carried out. The spatial memory of transgenic tau mice given zinc supplemented water as assessed by Morris Water Maze was significantly worse when compared with transgenic mice given lab water. Furthermore, wheel-running behavior was altered such that tau mice given zinc water began running later than tau mice given lab water. The nests of tau mice on zinc water were also significantly worse than those of tau mice on lab water. Open field results indicated that tau mice were faster than wildtype mice. No significant differences were determined in fear conditioning, however this may be explained by the fact that these mice were tested at 7 months of age and the amygdala is not affected until at least this age. These findings differ from those of Harris and colleagues (2012) who showed progressive tau pathology progression but no behavioral differences up to 16 months of age. However, this study selectively expressed tau in the entorhinal cortex only, which may explain the lack of behavioral effects. In Alzheimer's patients showing tau protein in the hippocampus spatial memory deficits are

typically seen, and as such continued development of this mouse model may be necessary.

To summarize, this study determined that zinc exacerbates the biochemical effects of tau protein. This should be a framework for future studies and drug development for any neurological disorder involving tau protein. Future work should consider a double transgenic model of Alzheimer's disease to determine the effect of zinc on amyloid beta and tau protein together. Furthermore, zinc chelation as a therapeutic may be a viable option for all tauopathies and should be explored further.

REFERENCES

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M.,…Muiswinkel, F. L. (2000). Inflammation and Alzheimer's Disease. *Neurobiology of Aging, 23*(3), 383-421.

Alonso, A. D. C., Grundke-Iqbal, I., & Iqbal, K. (1996). Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nature medicine*, *2*(7), 783-787.

Augustinack, J. C., Schneider, A., Mandelkow, E. M., & Hyman, B. T. (2002). Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. *Acta Neuropathology, 103*, 26-35. doi:10.1007/s004010100423

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta neuropathologica*, *82*(4), 239-259.

Bramblett, G. T., Goedert, M., Jakes, R., Merrick, S. E., Trojanowski, J. Q., & Lee, V. M. (1993). Abnormal tau phosphorylation at Ser 396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron*, *10*(6), 1089-1099.

Brewer, G. J. (2012). Copper excess, zinc deficiency, and cognitive loss in Alzheimer's disease. *BioFactors, 38*, 107-113.

Bush, A. I., Pettingell, W. H., Multhaup, G., d Paradis, M., Vonsattel, J. P., Gusella, J. F., ... & Tanzi, R. E. (1994). Rapid induction of Alzheimer A-beta amyloid formation by zinc. *Science*, *265*(5177), 1464-1468.

Cook, C., Kang, S. S., Carlomagno, Y., Lin, W. L., Yue, M., Kurti, A., ... & Rousseau, L. (2015). Tau deposition drives neuropathological, inflammatory and behavioral abnormalities independently of neuronal loss in a novel mouse model. *Human molecular genetics*, *24*(21), 6198-6212.

Deibel, M. A., Ehmann, W. D., & Markesbery, W. R. (1996). Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. *Journal of the neurological sciences*, *143*(1), 137-142.

Dumont, M., Stack, C., Elipenahli, C., Jainuddin, S., Gerges, M., Starkova, N. N., Yang, L., Starkov, A. A., & Beal, F. (2011). Behavioral deficit, oxidative stress, and mitochondrial dysfunction precede tau pathology in P301S transgenic mice. *FASEB Journal*, *25*, 4063-4072.

Flinn, J. M., Hunter, D., Linkous, D. H., Lanzirotti, A., Smith, L. N., Brightwell, J., Jones, B. F. (2005). Enhanced zinc consumption causes memory deficits and increased brain levels of zinc. *Physiology & Behavior, 83,* 793-803.

Frederickson, C. J., Burdette, S. C., Frederickson, C. J., Sensi, S. L., Weiss, J. H., Yin, H. Z., …Lippard, S. J. (2004). Method for identifying neuronal cells suffering zinc toxicity by use of a novel fluorescent sensor. *Journal of Neuroscience Methods, 139*, 79-89.

Frederickson, C. J., Kasarkis, E. J., Ringo, D., Frederickson, R. E. (1987). A quinolone fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. *Journal of Neuroscience Methods, 20,* 91-103.

Frederickson, C. J., Suh, S. W., Silva, D., Frederickson, C. J., Thompson, R. B. (2000). Importance of zinc in the central nervous system: The zinc containing neuron. *Journal of Nutrition, 130,* 1471-1483.

Götz, J., Chen, F., Barmettler, R., & Nitsch, R. M. (2001). Tau filament formation in transgenic mice expressing P301L tau. *Journal of Biological Chemistry*, *276*(1), 529-534.

Hancock, S. M., Finkelstein, D. I., & Adlard, P. A. (2014). Glia and zinc in ageing and Alzheimer's disease: a mechanism for cognitive decline? *Fronteirs in Aging Neuroscience, 6(*137), 1-6.

Harris, J. A., Koyama, A., Maeda, S., Ho, K., Devidze, N., Dubal, D. B., ... & Mucke, L. (2012). Human P301L-mutant tau expression in mouse entorhinal-hippocampal network causes tau aggregation and presynaptic pathology but no cognitive deficits. *PloS one*, *7*(9), e45881.

Huang, Y., Wu, Z., Cao, Y., Lang, M., Lu, B., & Zhou, B. (2014). Zinc binding directly regulates tau toxicity independent of tau hyperphosphorylation. *Cell Reports, 8*(3), 831-842.

Iqbal, I., Iqbal, K., Tung, Y., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeleton pathology. *Proceedings of the National Academy of Sciences, 83*, 4413-4917.

Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y. C., Zaidi, M. S., & Wisniewski, H. M. (1986). Microtubule-associated protein tau. *The Journal of Biological Chemistry, 261*(13), 6084-6089.

Kanno, T., Tsuchiya, A., & Nishizaki, T. (2014). Hyperphosphorylation of tau at ser396 occurs in the much earlier stage than appearance of learning and memory disorders in 5XFAD mice. *Behavioural Brain Research, 274*, 302-306.

Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacharaju, P., Van Slegtenhorst, M., ... & Duff, K. (2000). Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nature genetics*, *25*(4), 402-405.

Lindwall G. and Cole R. D. (1984) The purification of tau protein and the occurrence of two phosphorylation states of tau in brain. *J. biol. Chem.* 259, 12241–12245.

Liu, L., Drouet, V., Wu, J. W., Witter, M. P., Small, S. A., Clelland, C., & Duff, K. (2012). Trans-synaptic spread of tau pathology in vivo. *PloS one, 7*(2), e31302.

Lovestone, S., & Reynolds, C. H. (1997). The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. *Neuroscience*, *78*(2), 309-324.

Maret, W., & Sandstead, H. H. (2006). Zinc requirements and the risks and benefits of zinc supplementation. *Journal of Trace Elements in Medicine and Biology, 20*, 3-18.

Mondragon-Rodriguez, S., Perry, G., Munoz, L., Acevedo-Aquino, M. C., & Williams, S. (2014). Phosphorylation of tau protein at sites Ser396-404 is one of the earliest events in Alzheimer's disease and Down syndrome. Neuropathology and Applied Neurobiology, 40, 121-135. doi:10.1111/nan.12084

Nuttall, J. R., & Oteiza, P. I. (2014). Zinc and the aging brain. *Genes and Nutrition, 9*, 1-11.

Pepersack, T., Rotsaert, P., Benoit, F., Willems, D., Fuss, M., Bourdoux, P, & Duchateau, D. (2001). Prevalence of zinc deficiency and its clinical relevance among hospitalized elderly. *Archives of Gerontology and Geriatrics*, *33*(3), 243-253.

Prasad, A. S., Fitzgerald, J. T., Hess, J. W., Kaplan, J., Pelen, F., & Dardenne, M. (1993). Zinc deficiency in elderly patients. *Nutrition, 9*(3), 218-224.

Railey, A. M., Smith, L. N., Micheli, T. L., Morgan, K., Fitzgerald, A. A., VeeJay, M., ... & Chrosniak, L. D. (2006). *Cognitive Effects of Long-term Enhanced Dietary Zinc Consumption: Modulation by Copper* (Doctoral dissertation, George Mason University).

Railey, A. M., Micheli, T. L., Wanschura, P. B., Flinn, J. M. (2010). Alterations in fear response and spatial memory in pre- and post-natal zinc supplemented rats: Remediation by copper. *Physiology & Behavior, 100*, 95-100.

Selden S. C. and Pollard T. D. (1983) Phosphorylation of microtubule associated proteins regulates interaction with actin filaments. *J. biol. Chem.* 258**,** 7064–7071.

Shrag, M., Crofton, A., et al. (2011). Effect of cerebral amyloid angiopathy on brain iron, copper, and zinc in Alzheimer's disease. *Journal of Alzheimer's Disease, 24*(1), 137-149.

Sun, A., Liu, M., Nguyen, X. V., & Bing, G. (2003). p38 MAP kinase is activated at early stages in Alzheimer's disease brain. *Experimental Neurology, 183*, 394-405. Doi:10,1016/S0014-4886(03)00180-8

Sun, X. Y., Wei, Y. P., Xiong, Y., Wang, X. C., Xie, A. K., Wang, X. L.,…Wang, J. Z. (2012). Synaptic released zinc promotes tau hyperphosphorylation by inhibition of protein phosphatase 2a (pp2a). *Journal of Biological Chemistry, 287*, 11174-11182.

Takeda, A., Tamano, H., Tempaku, M., Sasaki, M., Uematsu, C., Sato, S., ... & Bush, A. I. (2017). Extracellular Zn2+ Is Essential for Amyloid β1–42-Induced Cognitive Decline in the Normal Brain and Its Rescue. *Journal of Neuroscience*, *37*(30), 7253-7262.

Voss, K., Harris, C., Ralle, M., Duffy, M., Murchison, C., & Quinn, J. F. (2014). Modulation of tau phosphorylation by environmental copper. *Translational neurodegeneration*, *3*(1), 24.

Wang, J. Z., Xia, Y. Y., Grundke-Iqbal, I., & Iqbal, K. (2013). Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. *Journal of Alzheimer's Disease*, *33*(s1), S123-S139.

Weingarten, M. D., Lockwood, A. H., Hwo, S., & Kirschner, M. W. (1975). A protein factor essential for microtubule assembly. *Proceedings of the National Academy of Sciences*, *72*(5), 1858-1862.

Woodroofe, C. C., Masalha, R., Barnes, K. R., Frederickson, C. J., & Lippard, S. J. (2004). Membrane-permeable and impermeable sensors of the Zinpyr family and their application of imaging of hippocampal zinc in vivo. *Chemistry & Biology, 11*, 1669-1666.

Xiong, Y., Luo, D. J., Wang, X. L., Qiu, M., Yang, Y., Yan, X.,…Liu, R. (2015). Zinc binds to and directly inhibits protein phosphatase 2A in vitro. *Neuroscience Bulletin, 31*, 331-337.

Xiong, Y., Jing, X. P., Zhou, X. W., Wang, X. L., Yang, Y., Sun, X. Y., Qiu, M., Cao, F. Y., Lu, Y. M., Liu, R., & Wang, J. Z. (2013). Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation. *Neurobiology of Aging*, *34*, 745-756.

Zhou, F., Chen, S., Xiong, J., Li, Y., & Qu, L. (2012). Luteolin reduces zinc-induced tau phosphorylation at Ser262/356 in an ROS-dependent manner in SH-SY5Y cells. *Biological trace element research*, *149*(2), 273-279

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