Evaluation of Aging and Genetic Mutation Variants on Tauopathy

Amber M. Tetlow

University of South Florida

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Evaluation of Aging and Genetic Mutation Variants on Tauopathy

by

Amber M. Tetlow

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Aging Studies
College of Community and Behavioral Sciences
University of South Florida

Co-Major Professor: Dave Morgan, Ph.D.
Co-Major Professor: Ross Andel, Ph.D.
Paula Bickford, Ph.D.
Alyssa Gamaldo, Ph.D.
Cathy McEvoy, Ph.D.
Brent Small, Ph.D.

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ABSTRACT

Alzheimer’s disease (AD) is characterized by amyloid β plaques and neurofibrillary tau tangles (NFTs). While research has demonstrated amyloid pathology occurs prior to tau pathology, or tauopathy, tau has proven to be more toxic. Tauopathy is associated with cognitive declines and neurodegeneration. These findings have highlighted the importance of further understanding tauopathy. In the progression of tauopathy, there is an observable immune response that can be measured by glial cells such as microglia. Activated microglia are known to exacerbate tauopathy rather than reducing the pathology. Research has indicated that with increased age there is an increased risk for AD-related tauopathy and a more reactive, or primed, immune response. Therefore, it is important to further understand how tauopathy and immune markers change in respect to age to potentially identify critical periods that might be advantageous for future interventions. This dissertation is aimed at understanding the effects of age and genetic mutant variants of tau in different animal models of tauopathy over three different studies.

In study one, a commonly used transgenic model of tauopathy, rTg4510, was studied to examine how markers of tauopathy and tauopathy-related pathology differ over the life course. Regression analyses were conducted to determine the best models of fit (linear vs non-linear models of fit) for each marker. Results indicated that not all the markers of pathology in this model progress at the same rate or in the same manner. However, there was an overall increase in pathological events with increased age; especially in histological sections and the detergent insoluble homogenate fraction that contains pathological aggregates.
In study two, age differences between young, middle-aged, and old animals that received either AAV9 GFP or AAV9 tau\textsuperscript{P301L} intracranial injections were examined. Two-way ANOVAs and two-way repeated measures ANOVAs were conducted to determine group differences on measures of tauopathy, tauopathy-related markers of pathology including immune activation and neurodegeneration, and behavioral assessments of motor and cognitive impairments. These results indicated the old mice had higher levels of early phosphorylated tau, neurofibrillary tau tangles (NFTs), and reactive immune activation. Behavioral assessments evidenced a reduction in performance in the old animals irrespective of the injection group. However, only the younger GFP injected mice (young and middle-aged) were able to demonstrate mastery of the memory task. The old GFP and tau\textsuperscript{P301L} injected mice were not able to achieve the historical learning criterion in the memory task.

In study three, viral constructs expressing different tau variants were employed including, AAV9 tau\textsuperscript{P301L}, AAV9 tau\textsuperscript{R406W}, AAV9 tau\textsuperscript{wild-type}, and AAV9 GFP intracranial injections were conducted in middle aged mice to identify whether tau mutations associated with human disease could produce a translational model of tauopathy. One-way ANOVAs and one-way repeated measures ANOVAs were conducted for measures of tauopathy, tauopathy-related pathology including immune activation and neurodegeneration, and behavioral assessments to determine any motor or cognitive deficits. The results indicated that both tau\textsuperscript{P301L} and tau\textsuperscript{wild-type} created models of tauopathy that include declines in a behavioral memory task. However, these two viruses displayed different mechanisms that warrant further investigation. Tau\textsuperscript{P301L} demonstrated the highest levels of insoluble tau and NFTs, similar to the rTg4510 model, while tau\textsuperscript{wild-type} had the highest levels of histological phosphorylated tau, soluble tau, and exhibited the greatest amount of hippocampal atrophy.
This body of work demonstrates the importance of age in both transgenic and viral models of tauopathy. As demonstrated by studies one and two, there are changes that occur in animal models that can demonstrate a progression of pathology. This progression is critical as translational models of AD-related tauopathy are imperative for furthering the knowledge of tau and potential treatments. Additionally, the viral models validate the need to understand earlier events in the progression of pathology. The tau$^{P301L}$ and tau$^{\text{wild-type}}$ viruses both modeled tauopathy. However, these viruses, did so in distinct ways that warrant further investigation to determine if there are differences early in the progression of pathology that are driving the observed differences found in age and/or viral differences.
CHAPTER ONE:

BACKGROUND

The increase in the aging population is in part due to the medical advancements that have allowed for the identification of treatments and preventative approaches for many of the leading causes of death (Johnson, Hayes, Brown, Hoo, & Ethier, 2014). Currently, Alzheimer’s disease (AD) is the sixth leading cause of death in the United States and lacks any FDA approved treatments to reverse or halt AD pathology (Cummings, Lee, Ritter, Sabbagh, & Zhong, 2019; Cummings, Morstorf, & Zhong, 2014; Huang, Chao, & Hu, 2020). There are two subgroups of AD, familial AD, and sporadic AD. Most AD cases are sporadic AD where there is no confirmed etiological cause compared to familial AD that is linked to genetic mutations. Currently, increased age is the greatest known risk factor for a sporadic AD diagnosis. While there are no disease-modifying treatments, research has identified key biological indicators of AD that include extracellular amyloid β plaques and intracellular neurofibrillary tau tangles (NFTs).

Research has indicated amyloid pathology is one of the early events in the progression of AD (Bush, Beyreuther, & Masters, 1992; Podlisny, Tolan, & Selkoe, 1991; Selkoe, 1991; Tanaka et al., 1992). One of the first mutations associated with familial AD was the genetic mutation of the amyloid precursor protein that increases production of amyloid β plaques by increasing the length of the amyloid β peptide (Goate et al., 1991). These findings contributed to the amyloid cascade hypothesis that posits the production of amyloid β is the pivotal event to AD pathology (Hardy & Higgins, 1992). It has been discovered that the pathological changes in
amyloid β can occur as early as 20 years prior to the onset of symptoms (H. Braak, Braak, Bohl, & Reintjes, 1996; Villemagne et al., 2008). Historically, this has led many clinical trials to focus on the reduction and clearance of amyloid β (Huang et al., 2020). Surprisingly, these clinical trials have demonstrated not only limited cognitive improvements, but limited amyloid clearance and prevention of further degeneration (Delrieu, Ousset, Voisin, & Vellas, 2014; Karran & Hardy, 2014; Salloway et al., 2009; Salloway et al., 2014). Given the demonstrated lack of overall efficacy of these clinical trials, some researchers have suggested future efforts to focus on additional aspects of the disease such as tau (Kametani & Hasegawa, 2018; Ricciarelli & Fedele, 2017). Numerous studies have identified that tau pathology, or tauopathy, is strongly related to neurodegeneration and cognitive deficits (Bejanin et al., 2017; Gordon et al., 2018; Hanseeuw et al., 2019; Malpas, Sharmin, & Kalincik, 2020). This supports the importance of studies to further understand the mechanisms of tauopathy.

**Tau**

Tau is a necessary microtubule-associated protein that is primarily located in the axon of neurons. Tau is crucial for the stabilization of the cytoskeleton, aiding in axonal transportation, and synaptic plasticity (Avila, Lucas, Perez, & Hernandez, 2004; Drubin & Kirschner, 1986; Y. Wang & Mandelkow, 2015). There are six isoforms of tau (Buée, Bussiere, Buée-Scherrer, Delacourte, & Hof, 2000). These different isoforms are generated by alternative splicing of exons 2, 3, and 10 of the human tau MAPT gene. Exons 2 and 3 are located in the amino terminus projection domain. The projection domain functionality determines the distance between the microtubules (Chen, Kanai, Cowan, & Hirokawa, 1992). The alternate splicing for the projection domain includes the presence of both exons 2 and 3, referred to as N1 and N2 (2N), N1 only (1N), or lacks both N1 and N2 (0N). Exon 10 is located in the microtubule
binding domain and is responsible for binding to the microtubules and to other proteins (F. Liu & Gong, 2008). Alternate splicing of exon 10 can result in either 3 (3R) or 4 (4R) microtubule binding repeat domains. Many of the detrimental mutations observed in tauopathies other than AD occur in exon 10 (F. Liu & Gong, 2008). In AD, both 3R and 4R tau have been observed in NFTs (Espinoza, De Silva, Dickson, & Davies, 2008; Rosenberg, Ross, Feinstein, Feinstein, & Israelachvili, 2008). Moreover, there appears to be a shift in the 4R to 3R ratio in the progression of AD, wherein the 4R tau predominance transitions to 3R (F. Liu & Gong, 2008; Uchihara, 2014).

Once tau is synthesized it can undergo a variety of post-translational modifications, but the most widely studied in coordination with AD has been phosphorylation. While phosphorylation is required for several different important biological mechanisms, when it becomes hyperphosphorylated, tau often results in pathological conformational changes (Dubey, Ratnakaran, & Koushika, 2015; Y. Wang & Mandelkow, 2015). These conformational changes result in a severe reduction in tau’s binding affinity to the microtubules. With a reduction in microtubule binding, tau begins to accumulate and migrate to the somatodendritic compartment (Gendreau & Hall, 2013). In addition, hyperphosphorylated tau is more resistant to degradation and has a propensity to sequester additional tau from the axon. Once additional tau is sequestered, tau begins to form aggregates that are referred to as paired helical filaments (PHFs). Research has indicated serine 396 as an important phosphorylation epitope of tau for the formation of PHFs (Bramblett et al., 1993). As PHFs are resistant to degradation and continue to aggregate, this results in the destabilization of the cytoskeleton and the formation of argyrophilic NFTs (H. Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredici, 2006). The structural formation of NFTs contributes to cellular death as the neuron is no longer able to transport vital
material within the cell required for survival. Once the neuron dies, an extracellular “ghost” tangle” is left behind that still retains argyrophilic properties (F. Braak, Braak, & Mandelkow, 1994; Endoh, Ogawara, Iwatsubo, Nakano, & Mori, 1993; Uchihara, 2014; Uchihara, Hara, Nakamura, & Hirokawa, 2012).

**Tau and Inflammation**

In recent decades research has proven that the brain is not “immune privileged” as once assumed. Aging research has identified there is an increase in the baseline inflammatory status with increased age (Franceschi et al., 2000; Franceschi & Campisi, 2014; Franceschi et al., 2007). This priming effect for pro-inflammatory mediators, generates more reactivity with age. In addition, there are deficiencies in anti-inflammatory mechanisms with age, so it takes longer for the body to recover from an inflammatory response. However, in age-related diseases such as AD, inflammation is amplified by the chronic presence of pathology (Akiyama et al., 2000; Nizami, Hall-Roberts, Warrier, Cowley, & Di Daniel, 2019; F. Su, Bai, & Zhang, 2016; W. Y. Wang, Tan, Yu, & Tan, 2015; Zhang, Li, Ng, & Song, 2015).

The cells responsible for the inflammatory immune response within the central nervous system are glial cells. Two of the glial cell types that have been examined in relation to AD are microglia and astrocytes. Microglia are constantly sampling the environment which allows them to quickly respond to homeostatic changes. To respond to changes in the environment, microglia recognize pathogens using pattern recognition receptors, activate phagocytic properties, and modulate additional pro-inflammatory factors (i.e., cytokines and chemokines) (Boche, Perry, & Nicoll, 2013; Norden & Godbout, 2013). Astrocytes release additional inflammation factors, but
also respond to injury, maintain ionic concentrations within and outside the cell, and regulate the endothelial cells of the blood brain barrier (Abbott, Rönnbäck, & Hansson, 2006; Walz, 2000).

Interestingly there appears to be a paradoxical relationship for inflammation and AD pathology. Immune activation generally can stimulate amyloid β clearance, but has consistently been reported to exacerbate tauopathy (Ghosh et al., 2013; Herber et al., 2007; Joly-Amado et al., 2020; Lee et al., 2010). This paradoxical relationship highlights the importance of further understanding tau in relation to inflammation.

Clinical Alzheimer’s Disease Tauopathy

AD tauopathy progresses in a temporal manner that begin in the transentorhinal cortex and ultimately encompasses critical cortical and subcortical areas. The Braaks have defined six stages of AD-related tauopathy (H. Braak & Braak, 1991; H. Braak & Braak, 1995). Stage I includes the mild formations of NFTs in the transentorhinal cortex with NFTs spreading to the hippocampal areas in Stage II. Stage III is classified by NFTs projecting to other cortical areas and initial ghost tangles become evident in the transentorhinal areas. Stages IV and V entails continued projections to cortical regions and an increase in NFT severity. In the final stage, ghost tangles and NFTs are abundant throughout the cortical and subcortical areas coupled with neuronal loss.

These stages have remained a significant classification as clinical imaging has validated the stages (Davis, Schmitt, Wekstein, & Markesbery, 1999; Marquié et al., 2017; Schwarz et al., 2016). Cognitive researchers have identified that tauopathy is closely related to cognitive deficits (Swinford, Risacher, Charil, Schwarz, & Saykin, 2018). The most commonly reported cognitive deficit is memory. However, years before substantial deficits in global memory and activities of
daily living are noticed, changes in episodic and spatial memory can be identified (Blennow, de Leon, & Zetterberg; Dubois et al., 2007; Schindler et al., 2017; Villemagne et al., 2008; Wilson, Leurgans, Boyle, & Bennett, 2011). Episodic and spatial memory are hippocampal dependent constructs (Bäckman, Jones, Berger, Laukka, & Small, 2005; Burgess, Maguire, & O'Keefe, 2002; Glisky, 2007; Hedden, 2007; Serino & Riva, 2014). As evidenced by the Braak stages, the hippocampus is one of the brain structures that is impacted early in AD related tauopathy (H. Braak et al., 1996; H. Braak, Thal, Ghebremedhin, & Del Tredici, 2011). As the pathology burden continues, the clinical symptomology does as well. Clinical AD symptoms progress to retrograde amnesia, deficits in attention, executive function, language, and even motor function in advanced stages (Brugger, Monsch, Salmon, & Butters, 1996; Faust & Balota, 1997; Hehman, German, & Klein, 2005).

Animal Models of Tauopathy

Transgenic Models

Since biological pathology is evident many years before the onset of clinical symptoms, it is important to understand the biological mechanisms that occur in prodromal stages of AD. Therefore, animal models are commonly employed. One of the early tau transgenic lines was the JPNL3 P301L lineage, where the pathology is driven by the mouse prion promoter (Lewis et al., 2000). This lineage was formed after the identification of the 4R0N P301L tau mutation for frontotemporal dementia and parkinsonism chromosome 17 (FTD-17) (Hutton et al., 1998). Lewis et al. (2000) noted an increase of insoluble tau from 2 to 8 months of age, NFTs by 4.5 months, and evidence of inflammation. However, there was also pathology in the spinal cord
resulting in hindlimb paralysis by 10 months of age. This is a large confound for both aging studies and animal behavioral assessments.

In hopes of improving the JPNL3 P301L animal model, the rTg4510 line was created (Ramsden et al., 2005; Santacruz et al., 2005). This is a bigenic line that is created from breeding a mouse that harbors a tetracycline-operon-responsive element upstream of the 4R0N P301L mutation with another mouse containing the tetracycline-controlled transcriptional activator downstream of the CaMKIIα (calcium calmodulin kinase II subunit alpha) promoter (Santacruz et al., 2005). The tetracycline-controlled transcriptional activator allows for the suppression of mutant tau expression with the treatment of doxycycline. The CaMKIIα promoter predominately expresses pathology to the forebrain and cortex while preserving the spinal cord, and the animals do not experience the hindlimb paralysis of the previous tau model (Dutschmann et al., 2010; Santacruz et al., 2005). The seminal work by Ramsden et al. (2005) included time series experiments up to 10 months of age. NFTs and memory impairments begin to accumulate as early as 2.5 months, with cortical atrophy at 9 months. Furthermore, there was noted neuronal loss, and astrocyte activation. At 16 months there was evidence of atrophy and a 30% reduction in brain weight was noted. Further research has evidenced an upregulation of inflammatory pathways for this model (H. Wang et al., 2018; Wes et al., 2014).

**Viral Models**

While transgenic models have proven to be instrumental for many research discoveries, they generally produce an overabundance of pathology. For AD transgenic models, they generally produce pathology early in life which more closely mimics familial AD and not sporadic AD. Because the vast majority of AD diagnoses are sporadic and have no known
genetic component, transgenic models have less translational value for sporadic AD. Therefore, many researchers have employed the use of viral models of tauopathy. Many use adeno-associated viral vectors (AAV), which is a form of gene therapy that allows for the transfer of selected viral constructs to a region of interest (Daya & Berns, 2008). AAV is part of the parvovirus family but requires the presence of helper plasmids to ensure outer capsid assembly and allows for viral replication. Because AAV has an open reading frame where genes of interest can be inserted, it is commonly referred to as an empty cassette system. The open reading frame is flanked by inverted terminal repeats that allows for the insertion of 4.5kb of genomic material such as the gene of interest and any viral tags or regulatory elements that may enhance the viral expression (Loeb, Cordier, Harris, Weitzman, & Hope, 1999; Naso, Tomkowicz, Perry, & Strohl, 2017). There are many different AAV serotypes that target specific cells. AAV9 is a commonly used serotype that has a preference for neurons especially following intracranial injections (Dayton, Wang, & Klein, 2012).

Because of the preference for neuronal transduction, AAV9 has been used for the construction of viral models of tauopathy (Mustroph, King, Klein, & Ramirez, 2012; Vanderweyde et al., 2016). Research conducted by X. Liu et al. (2017) evaluated intracranial entorhinal cortex injections of AAV9 tau$^{\text{P301L}}$ and AAV9 green florescent protein (GFP) in three month old male C57BL/6 mice that were incubated for six weeks. GFP is commonly used as a control virus as this causes the infected cells to fluoresce green. There was evidence of tau expression that encompassed the cortex and hippocampus including phosphorylated epitopes of pSer199 and pSer396. Additionally, there was a reduction of neurons in the dente gyrus of the hippocampus, reduction of synaptic density measured by PSD-95, and a decline in a behavioral social transmitted food preference test (STFP). STFP is a hippocampal dependent food
preference test. Cook et al. (2015) examined differences between AAV1 tau$^{P301L}$ and the rTg4510 transgenic line to ensure tau expression. There was evidence of robust tau expression after 6 months of viral incubation after intracerebroventricular injections into newly born mouse pups. Further analysis found evidence of phospho-tau epitopes pSer396/404, pSer202, and pSer262/356. There were silver positive NFTs, with microglia and astrocyte activation when compared to AAV1 GFP injection. Jaworski et al. (2009) employed the use of AAV1/2-Tau$^{P301L}$ and AAV1/2-Tau$^{\text{wild-type}}$ along with a control AAV1/2 GFP using intracerebral injections. There was evidence that the tau$^{P301L}$ induced hippocampal thinning and both P301L and wild-type tau groups had neuronal loss by 3 weeks. When examining tau expression and phospho-tau epitopes including pSer202, pThr205, pThr231, pSer396, and pThr404 demonstrated that the AAV1/2 tau$^{P301L}$ had more tauopathy present when compared to tau$^{\text{wild-type}}$. Furthermore, there was evidence of microglia and astrocyte activation.

Using the background knowledge of current tauopathy research, this dissertation examined both transgenic and viral models of tauopathy over three different studies.

**Study One** examined how markers of pathology differ over the life course of the rTg4510 mouse model. To achieve this, tissues were collected from both male and female rTg4510 mice aged from two to twenty months. Histological and biochemical measures include markers of tauopathy, microglia, astrocytes, and indicators of neurodegeneration.

**Study Two** investigated the effect of age on a viral method of tauopathy after the induction AAV9 GFP and AAV9 tau$^{P301L}$ in 8-, 12-, and 16-month old male and female C57BL/6 mice. Injections were administered to bilateral hippocampi and anterior cortices. Behavioral assessments were conducted to evaluate any evidence of cognitive impairments at 3.5
months of viral incubation. Tissues were collected at 4 months and analyzed to evaluate the effect of age of onset on markers of tauopathy, immune activation, and neurodegeneration.

**Study Three** explored effects of different AAV9 tau variants that included tau\textsuperscript{wild-type}, tau\textsuperscript{P301L}, and tau\textsuperscript{R406W} with AAV9 GFP as a control virus. This was conducted to determine if one of these constructs may be more of a translational model of AD tauopathy. Injections were administered bilaterally to hippocampi and anterior cortices. At 3.5 months of viral incubation, behavioral assessments were conducted to determine any evidence of cognitive impairment based on the tau variant injection. Tissues were collected at 4 months post injection and analyses were conducted to determine group differences based on tau variant injection on measures of tauopathy, immune activation, and neurodegeneration.
CHAPTER TWO:

AGE SERIES EXAMINATION OF THE rTG4510 MODEL OF TAUOPATHY

Introduction

Tauopathy is one of the hallmark features of Alzheimer’s Disease (AD) and is closely related to cognitive decline and neuronal loss (Bejanin et al., 2017; Malpas et al., 2020). To date, age still proves to be the greatest risk factor for AD-related tauopathy and increased tau burden. In the progression of tauopathy, it is evident that there are measurable changes early in the phosphorylation process and research indicates this can be measured by the phospho-tau epitopes of serine199 and serine202 (Luna-Munoz, Chavez-Macias, Garcia-Sierra, & Mena, 2007; J. H. Su, Cummings, & Cotman, 1996). As pathology increases in severity, further phosphorylation occurs and the phospho-tau serine396 epitope (Kimura et al., 1996; Mondragón-Rodríguez, Perry, Luna-Muñoz, Acevedo-Aquino, & Williams, 2014). This later phospho-tau epitope has proven to be a critical residue for the formation of paired helical filaments (PHFs) which is an intermediary progression to the formation of neurofibrillary tau tangles (NFTs) (Bramblett et al., 1993).

Furthermore, in the aging process where there is an increased risk for AD pathology, there is also a general increase in pro-inflammatory homeostatic balance (Franceschi & Campisi, 2014). Research has demonstrated that this increase in inflammation that has proven to increase tau burden (Ghosh et al., 2013; Herber et al., 2007; Lee et al., 2010). Therefore, the examination
of immune activation with age is an important aspect to evaluate when examining tauopathy as this may be an advantageous avenue for future interventions.

The rTg4510 line has been extensively used as a model of tauopathy. This line expresses the 4R0N tau P301L mutation and expresses pathology in the cortex and hippocampus (Santacruz et al., 2005). Previous research has demonstrated that rTg4510 line exhibits age-dependent neurofibrillary tau tangles (NFTs), neuronal loss, behavioral impairments, and an inflammatory response (Ramsden et al., 2005; Santacruz et al., 2005). The seminal work of this transgenic line included short time course studies of tauopathy up to approximately 10 months of age (Ramsden et al., 2005; Santacruz et al., 2005). These studies observed NFTs as early as 2.5 months and significant reductions in brain weight by 5 months. Additional research has extended age differences to 13 and 15 months that indicate increased pathology with age (Blackmore et al., 2017; Ludvigson, Luebke, Lewis, & Peters, 2011). When examining age in the rTg4510 line, it is important to note that Ramsden et al. (2005) evidenced that this line can survive up to 20 months of age.

While there has been a general consensus of an increase of NFTs with age in the rTg4510 line, work by Dickey et al. (2009) found that not all phospho-tau epitopes increase in a linear fashion as demonstrated by an age series examination up to 10 months of age. This leads to further investigation to understand how multiple markers of varying degrees of tauopathy severity differs over the life course in respect to important hallmarks of tauopathy including phosphorylated tau epitopes, NFTs, inflammation, and indicators of neuronal loss and atrophy. To date, no study has provided a detailed age series examination of the rTg4510 line that has extended to 20 months of age. Furthermore, this is the first study to compare different models of fit to determine how rTg4510 pathology changes at increasing ages. Therefore, the purpose of
this study is to address how these critical measures of tauopathy differ at increasing age points in three aims.

**Aim One:** To investigate how markers of tauopathy differ at increasing ages, including measures of total tau, early and late indicators of phosphorylation, and NFTs up to 20 months of age.

*Aim one hypotheses:* Indicators of tauopathy including total tau, early and late phosphorylated tau, and NFTs will be most severe in the 20-month old mice.

**Aim Two:** To determine how markers of tau-related pathology, including immune activation and neurodegeneration differ at increasing ages.

*Aim two hypotheses:* Given the prior research of increasing inflammation with age, there will be an increase in inflammation with increased age. Furthermore, it is expected that there will be a continued linear decrease in brain weight, hippocampal volume, and synaptic density based on the atrophy observed in the early studies of this model.

**Aim Three:** To examine how changes in tauopathy and other markers associated with tauopathy are related.

*Aim three hypotheses:* Indicators of tauopathy will be positively related to inflammation as the research indicates tau burden increases with inflammation. Furthermore, tau will be negatively related to neurodegeneration because we know tau is associated with degeneration and this will be most severe at 20 months of age.
Method

Animals

The rTg4510 line is a bigenic line bred by crossing one mouse line that contains both the 4R0N P301L tau mutation and the tetracycline-operon responsive element with another line that expresses the tetracycline-controlled transcriptional activator that is driven by the CaMKIIα promoter. This promoter restricts tauopathy predominately to the forebrain and extends to the rest of the cortex and hippocampus. This avoids spinal expression and subsequent paralysis seen in other mutant tau transgenic lines. Both male \((n=27)\) and female \((n=26)\) rTg4510 mice were used. Some mice born in this breeding lack both transgenes and are referred to as non-transgenic (ntg). Ntg mice were used as negative controls. Animals were kept on a 12-hour light cycle and were provided food water *ad libitum*. All animal protocols were conducted and approved by the Institutional Animal Care and Use Committee of the University of South Florida.

Tissue Collection

Mice were aged between 52 and 610 days of age until tissue collection. The mice were anesthetized with 0.1mL/10g Somnasol (0.25mL in 10mL of H₂O) and placed on a heating pad to maintain body heat to reduce tau phosphorylation caused by decreased body temperature during anesthesia (Planel et al., 2007). Animals were perfused transcardially with 25mL of 0.9% normal saline solution. Once the animals were perfused, the brains were immediately extracted. The brains were placed on an iced stage and the right hemisphere was dissected into the anterior region of the cortex (ACX), posterior region of the cortex (PCX), and the hippocampus (HPC). The brain regions were immediately frozen on dry ice. Brain region specimens were stored at -
80°C until homogenization for biochemical analysis. The left hemisphere was immediately immersed in 20mL of freshly prepared 4% paraformaldehyde (pH 7.4) for 24 hours at 4°C. After 24 hours, the left hemispheres were cryoprotected, where each brain was placed successively in 10, 20, and 30% sucrose for 24 hours. The brain hemispheres were stored at 4°C until sectioned. Brains were horizontally sectioned with a freezing microtome at a 25μm thickness and placed into Dulbecco’s phosphate buffered saline with 10 mM sodium azide solution (7.4 pH) except for every 8th section that was sectioned at 50μm thickness for hippocampal volumetric analysis.

**Brain Homogenization and Biochemical Analysis**

The PCX was added to a 10v/w RIPA (50mM Tris-Cl, 150nM NaCl, 1% NP40, 0.1% SDS, 0.05% sodium deoxycholate) buffer with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P8340), phenylmethanesulfonyl fluoride (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P7626), and phosphate inhibitor cocktails II and III (Sigma Aldrich, St. Louis, MO, USA; Cat. Nos. P5726 & P0044, respectively). Samples were homogenized first by a rotating mechanical pestle and then by sonication. Once sonicated, a consistent aliquot was removed from each sample and centrifuged at 50,000g for 1 hour at 4°C. The remaining crude homogenate was stored at -80 °C until analysis. Once the centrifugation was complete, the supernatant was removed and stored as the detergent soluble fraction, and the detergent insoluble pellet was kept for formic acid processing. Formic acid processing was conducted by adding a fifth of the total sample volume of 70% formic acid to each pellet and homogenized by a rotating mechanical pestle and sonicated again. After sonication, the samples were gently rotated for 30 minutes. 15.1M NaOH was added to neutralize formic acid samples. Once neutralized, 1M Tris-Base (pH 7.6) was added to a 1:2 ratio to the homogenate volume. Individual insoluble samples
were adjusted to pH of 7. Protein concentrations for crude, supernatant, and formic acid fractions were determined by Pierce BCA protein assays.

For western blot analysis, equal amounts of protein were added to each well (10ug/well) on Bio-Rad 4-20% polyacrylamide Criterion™ TGX Stain-Free Gels (Bio-Rad, Hercules, Cali; Cat. No. 5678094) and electrophoresed with tris glycine running buffer (Bio-Rad, Hercules, Cali; Cat. No. 1610732). Once the run was complete, gels were placed on Bio-Rad ChemiDoc MP imaging trays and were UV activated. UV activation was completed by using the imager’s UV activation function, which allows the compounds within the gel to bind to the proteins in the sample to display the total protein to be quantified for normalization of small differences in protein loading. After UV activation, the gels were transferred to 0.2μm midi nitrocellulose membranes (Bio-Rad, Hercules, Cali; Cat. No.1704159) using Bio-Rad Trans-Turbo Transfer System (Bio-Rad, Hercules, Cali; Cat. No.12003154). Membranes were blocked in 5% nonfat dry milk blocking buffer for 45 minutes and then incubated in the respective primary antibody for overnight incubation at 4˚C (Table 1). The following morning membranes were washed 3 times in tris buffered saline (TBS, (50 mM Tris-Cl, pH 7.6; 150 mM NaCl, pH 7.6) plus 0.07% tween-20 for 10 minutes per wash and then incubated in the corresponding secondary antibody for 1 hour. Finally, the membranes were washed 3 additional times in TBS+0.07% tween-20 for 10 minutes. Blots were imaged using Bio-Rad ChemiDoc MP. The total lane of positive antigen signal was normalized to the total protein.

**Histopathology**

Tissues from both rTg4510 and ntg mice were placed in free-floating multi-sample staining trays for each marker. The trays were placed in an endogenous peroxidase blocking
solution (10% methanol, 3%H$_2$O$_2$ in PBS). The tissues were washed 3 times with PBS and then placed in permeabilizing solution (0.2% lysine, 1% Triton X100, 4% normal goat serum in PBS). Tissues were then incubated overnight in the appropriate antibody (Table 1). The following morning, tissues were washed with PBS and incubated for 2 hours in the corresponding biotinylated secondary antibody. This step was excluded if the primary antibody was biotinylated. After the two-hour incubation period, tissues were washed with PBS and placed in Vectastain® Elite® ABC kit solution (Vector Laboratories, Burlingame, CA; cat. no. PK-6100) to enhance the enzyme complex. Again, tissues were washed with PBS for 2 washes and the final wash was in TBS. Following the TBS wash, tissues were placed in a color development solution (0.05% DAB, 0.5% Ni$^{2+}$, .03% H$_2$O$_2$) for 5 minutes. To stop color development, tissues were washed with TBS for one wash and then 2 PBS washes. Upon the completion of staining, tissues were mounted on microscope slides (Fisher Scientific, Waltham, MA; cat. no. 1255015), dehydrated, and cover slipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA; cat. no. 13512).

For Gallyas and cresyl violet staining, tissue sections were first mounted on microscope slides and dried overnight. Gallyas staining is a silver stain that identifies NFTs (Uchihara, 2007). On the day of Gallyas staining, sections were briefly rehydrated and then placed in a pretreatment solution (5% periodic acid). After a series of washes, the slides were immersed in a silver enhancing solution (10% potassium iodide, 4% NaOH, 0.035% silver nitrate) for one minute before incubating for 10 minutes in a 0.5% acetic acid solution. Slides were then placed in a working developer solution (2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.5% tungstosilic acid, 0.2% formaldehyde) for approximately 10 minutes. Upon development, slides were washed with 0.5% acetic acid. Then slides were immersed in a gold
toning solution (0.1% gold chloride solution) followed by an additional water wash. Then slides were incubated in a solution to remove traces of unconjugated silver salts (0.1% sodium thiosulphate solution) and a final water wash. Slides were immediately dehydrated and coverslipped.

The cresyl violet stain was conducted on pre-mounted 50μm tissues. On the day of the stain, slides were briefly rehydrated and then placed in a 0.05% cresyl violet dye solution. After staining, slides were briefly placed in an acetic acid water solution (pH 3.5) and rinsed in water. Slides were then dehydrated and coverslipped with DPX mounting medium (Electronic Microscopy Sciences Cat. No. 13512). Once the DPX was dried, slides were scanned using ZEISS Axio Scan.Z1. Scanned slides were analyzed using NearCyte tissue analysis IAE software (created by Andrew Lesniak). The cortex and hippocampus regions were identified and immunopositive pixels above a user-defined threshold were measured. The threshold parameters were derived using several sections with light and dark staining and held constant throughout the remaining analysis. The fraction of the total region occupied by immunopositive pixels was calculated. The values for each slide were then averaged together and for each region (ACX, PCX, and HPC). Cresyl violet staining was conducted to measure hippocampal volume using the method of Cavalieri.

**Statistical Analyses**

Statistical analyses were conducted using IBM SPSS Statistics 25 (Armonk, New York, USA) and figures were created with GraphPad Prism 8 (La Jolla, CA, USA). The average of fractional positive stained area for immunohistochemistry, Gallyas staining, hippocampal volume, and western signal intensities normalized to total protein were z-scored to be able to
compare across methodologies. Any data points that were greater than 3 standard deviations from the mean were considered statistical outliers and removed from analysis. Only four datapoints were excluded and this includes one for detergent insoluble total tau (HT7), detergent insoluble pSer199/202-tau, histological total tau (HT7), and histological pSer396-tau. Regression analyses were used to compare linear, quadratic, and cubic regressions to determine the best model of fit for each marker throughout the lifespan of rTg4510 mice. The $F$ change statistic was used to determine significant differences between linear, quadratic, and cubic models of fit. In addition, $R^2$ was used to determine the variance explained by age. Furthermore, Pearson correlations were used to determine the relationship between histological tau markers with inflammatory and neurodegenerative markers. To determine if any significant correlations were influenced due to the markers being largely age-dependent, partial correlations were conducted to control for age.

**Results**

**Aim One: How Markers of Tauopathy Differ with Increased Age**

*Neurofibrillary tangles*

As the data from different markers against age in the rTg4510 mice were plotted, it was evident that not all markers had linear trajectories. As a result, it was decided to contrast linear equations with quadratic and cubic fit equations to determine if the nonlinear fits were statistically superior. For Gallyas staining, a silver stain believed to detect NFTs, all three models of fit were significant (linear, $F(1,36) = 89.267, p < .001; R^2 = .713$, quadratic, $F(2,35) = 45.004, p < .001; R^2 = .720$, and cubic, $F(3,34) = 29.878, p < .001; R^2 = .725$). All three models of fit and the individual data points are included in Figure 1. The nonlinear fits failed to improve upon the
linear model (quadratic \( F \text{ change (1,35)} = .925, p = .343 \)) and cubic \( F \text{ change (1,35)} = 1.130, p = .295 \). Notably, Gallyas was the marker that had the most variance explained by age \( (R^2 = .713) \). Because the linear fit worked as well as the curvilinear models, it is believed that there is a linear increase in Gallyas staining with age in the rTg4510 model.

**Histological tau**

For histological tau markers (Figure 2), there was an increase in the amount of staining across the markers until approximately twelve months of age. At twelve months, HT7 staining, a marker of total tau, peaks and then begins to decline. The linear model of fit failed to reach significance, \( (F(1,31) = 2.586, p = .118) \), while the non-linear models were significant (quadratic, \( F(2,30) = 9.879, p = .001; R^2 = .397 \)) and cubic, \( F(3,29) = 6.367, p = .002; R^2 = .397 \). Further analysis found that the non-linear models were not significantly different \( (p = .971) \). In contrast to HT7, pSer396-tau exhibited a plateau at twelve months of age. Even though all three models of fit were significant (linear, \( F(1,33) = 15.111, p < .001; R^2 = .314 \), quadratic, \( F(2,32) = 12.078, p < .001; R^2 = .430 \), and cubic, \( F(3,31) = 7.816, p = .001; R^2 = .431 \)), the non-linear models of fit (quadratic, \( F \text{ change (1,32)} = 6.518, p = .016 \)) and cubic, \( F \text{ change (1,32)} = 6.520, p = .016 \) significantly improved the linear model. A further examination verified that the nonlinear models of fit were not significantly different \( (p = .871) \), thus the relationship was concluded to be quadratic. This model of fit supported the plateau observed at twelve months of age for histological pSer396.

In contrast, all three models of fit (linear, \( F(1,32) = 11.868, p = .002; R^2 = .271 \), quadratic, \( F(2,31) = 6.774, p = .004; R^2 = .304 \), and cubic, \( F(3,30) = 4.370, p = .011; R^2 = .304 \)) were significant for AT8 (pSer199, 202-tau). While all models were significant, the nonlinear
models of fit (quadratic, $F$ change (1,31) = 1.495, $p = .231$ and cubic, $F$ change (1,31) = 1.466, $p = .235$) failed to improve the linear model. This model indicates an increasing linear relationship with age.

**Detergent soluble tau**

When analyzing the supernatant detergent soluble tau markers measured by western blot (Figure 3), it was evident that pSer396-tau plateaued at approximately twelve months of age, resembling histological pSer396-tau. All three models of fit (linear, $F(1,47) = 24.115, p < .001$; $R^2 = .339$, quadratic, $F(2,46) = 16.935, p < .001$; $R^2 = .424$, and cubic, $F(3,45) = 11.515, p < .001$; $R^2 = .434$), were significant, with the non-linear models of fit improving the model (quadratic ($F$ change (1,46) = 6.787, $p = .012$) and cubic ($F$ change (1,46) = 7.360, $p = .009$). With further analysis, the non-linear models were not significantly different from one another ($p = .373$). In comparison to histological HT7 a measurement of total tau, detergent soluble HT7 decreased linearly with age, ($F(1,47) = 4.433, p = .041$). This linear relationship is demonstrated by the nonlinear models of fit failing to reach significance (quadratic, $F(2,46)= 2.384, p=.103; R^2 = .094$ and cubic, $F(3,45) = 2.423, p = .078; R^2 = .139$). Soluble pSer199/202-tau shares a phosphorylated epitope with histological AT8. Like AT8, all three models of fit were significant for detergent soluble pSer199/202-tau (linear, $F(1,45) = 50.012, p < .001$; $R^2 = .526$, quadratic, $F(2,44) = 24.453, p < .001; R^2 = .526$, and cubic, $F(3,43) = 19.985, p < .001; R^2 = .582$).

Additionally, the nonlinear models failed to improve the linear model (quadratic, $F$ change (1,44) = 5.789, $p = .958$, and cubic, $F$ change (1,44) = .120, $p = .731$). However, in contrast with histological AT8, soluble pSer199/202-tau declined with age.
**Detergent insoluble tau**

When examining the detergent insoluble (formic acid) fraction, tau markers measured by western blot (Figure 4), the markers changed in a similar manner as histological markers. For insoluble HT7, the linear model was not significant \((F(1,47) = 2.896, p = .095)\); whereas, both the non-linear models (quadratic, \(F(2,46) = 6.799, p = .003; R^2 = .228\), and cubic, \(F(3,44) = 4.437, p = .008; R^2 = .228\)) were significant. Insoluble HT7 resembled histological HT7, where the marker peaked at twelve months of age and then declined. Correlational analysis revealed a positive significant relationship between histological and insoluble HT7 \((r = .426, p = .024)\). In contrast, for detergent soluble pSer199/202-tau all three models of fit were significant (linear, \(F(1,48) = 10.180, p = .003; R^2 = .175\), quadratic, \(F(2,47) = 5.033, p = .010; R^2 = .176\), and cubic, \(F(3,46) = 3.318, p = .028; R^2 = .178\)), with the nonlinear fits failed to improve the linear model (quadratic, \(F\text{ change}(1,47) = 0.080, p = .779\) and cubic, \(F\text{ change}(1,47) = 0.101, p = .752\)).

Insoluble pSer199/202-tau was comparable to histological AT8, such that, they both increased linearly with age \((r = .436, p = .016)\). For insoluble pSer396-tau, all three models of fit were significant (linear, \(F(1,48) = 37.596, p < .001; R^2 = .439\), quadratic, \(F(2,47) = 19.001, p < .001; R^2 = .447\), and cubic, \(F(3,46) = 12.858, p < .001; R^2 = .456\)). However, both the quadratic \((F\text{ change}(1,47) = .667, p = .418)\) and cubic \((F\text{ change}(1,47) = .849, p = .362)\) models failed to improve upon the linear model. This statistical analysis confirmed an increasing linear relationship with age for detergent insoluble pSer396-tau. Even though the histological pSer396 plateaued, and insoluble pSer396 continued to increase with age, the markers exhibited a positive correlation \((r = .397, p = .027)\).
Aim Two: How Additional Tau-Associated Markers of Pathology Differ with Increased Age

Inflammation

When inflammation markers were analyzed, there was a general increase with age (see Figure 5). For histological CD45, all of the models of fit (linear, \( F(1,44) = 34.366, p < .001; R^2 = .439 \), quadratic, \( F(2,43) = 18.898, p < .001; R^2 = .468 \), and cubic, \( F(3,42) = 14.802, p < .001; R^2 = .514 \)) were significant. However, both the nonlinear models (quadratic, \( \text{F change (1,43) } = 2.365, p = .131 \) and cubic, \( \text{F change (1,43) } = 3.194, p = .081 \)) failed to significantly improve the model fit. Histological Iba-1 was comparable to CD45, where all models were significant (linear, \( F(1,40) = 53.298, p < .001; R^2 = .571 \), quadratic, \( F(2,39) = 26.646, p < .001; R^2 = .577 \), and cubic, \( F(3,38) = 17.718, p < .001; R^2 = .583 \)) yet the nonlinear models (quadratic, \( \text{F change (1,39) } = .569, p = .455 \) and cubic, \( \text{F change (1,39) } = .419, p = .521 \)) failed to improve the model fit. Similar to the previous markers, histological MHCII also had significant models of fit across linear (\( F(1,34) = 18.214, p < .001; R^2 = .349 \)), quadratic, (\( F(2,33) = 10.058, p < .001; R^2 = .379 \)), and cubic (\( F(3,32) = 8.089, p < .001; R^2 = .431 \)) models. When the differences were examined, the quadratic (\( \text{F change (1,30) } = .506, p = .483 \)) and cubic (\( \text{F change (1,30) } = .014, p = .365 \)) models of fit failed to significantly improve the linear model. Once again, all three models of fit, (linear (\( F(1,37) = 46.386, p < .001; R^2 = .556 \)), quadratic, (\( F(2,36) = 33.260, p < .001; R^2 = .649 \)), and cubic, (\( F(3,35) = 21.905, p < .001; R^2 = .652 \)) were significant for histological GFAP. However, unlike the other immune markers, the quadratic (\( \text{F change (1,36) } = 9.490, p = .004 \)) and cubic (\( \text{F change (1,36) } = 9.920, p = .003 \)) models of fit significantly improved the linear model. While GFAP increased with age like the microglia markers, there was an evident plateau at approximately fourteen months of age.
Neurodegeneration

Across several neurodegenerative markers, it is apparent there was neurodegeneration with increased age (Figure 6). All three models of fit for brain weight were significant (linear, $F(1,58) = 13.263, p = .001; R^2 = .186$, quadratic, $F(2,57) = 7.599, p = .001; R^2 = .211$, and cubic, $F(3,56) = 5.183, p = .003; R^2 = .217$). When comparisons between the linear and nonlinear models of fit (quadratic, $F_{change}(1,57) = 1.762, p = .190$, and cubic, $F_{change}(1,56) = .487, p = .488$) were conducted, it was evident that the nonlinear models failed to improve upon the linear model. Just as with brain weight, the linear model of fit was significant for hippocampal volume, $(F(1,47) = 17.834, p < .001; R^2 = .275)$ along with both nonlinear models of fit (quadratic, $F(2,46) = 9.351, p < .001; R^2 = .289$, and cubic, $F(3,45) = 6.938, p = .001; R^2 = .316$). Both the quadratic ($F_{change}(1,46) = .904, p = .347$) and cubic, ($F_{change}(1,46) = 1.232, p = .273$) models failed to improve the model fit. Additionally, all models of fit were significant for histological NeuN, (linear, $F(1,42) = 32.920, p < .001; R^2 = .439$, quadratic, $F(2,41) = 16.080, p < .001; R^2 = .440$, and cubic, $F(3,40) = 11.253, p < .001; R^2 = .458$), where the nonlinear models (quadratic, $F_{change}(1,41) = .013, p = .909$, and cubic, $F_{change}(1,41) = .067, p = .797$) failed to improve the model fit. While the linear model was significant for total crude homogenate PSD95, $(F(1,47) = 95.239, p < .001; R^2 = .670)$, both the nonlinear models (quadratic, $F_{change}(1,46) = 19.978, p < .001; R^2 = .770$, and cubic, $F_{change}(1,46) = 21.918, p < .001; R^2 = .780$) significantly improved the model fit. However, the two models of nonlinear fits were not significantly different ($p = .151$). This model fit exhibited a decline in PSD95 that plateaued at approximately twelve months of age.
Aim Three: The Relationship of Tauopathy with Tau-Related Pathology Across Increased Ages

When the relationships between tau with inflammatory and neurodegenerative markers were examined, there appeared to be many significant relationships (Table 2). However, the most remarkable tau marker was Gallyas. Gallyas had moderate to strong positive (Iba-1, MHCII, GFAP, and CD45) and negative (brain weight, NeuN, PSD95, and hippocampal volume) correlations with both inflammation and degenerative markers. However, when controlling for age in partial correlations, the significant relationships that were observed in the Pearson correlations lost significance.

Discussion

The purpose of this study was to provide an extension of the age-series data of the rTg4510 mouse line to 20 months of age. Furthermore, this study sought to examine how markers of pathology differ over the life course in three different aims. The first aim addressed how markers of tauopathy differ over the life course. This study demonstrated not all markers of tauopathy in rTg4510 mouse model follow the same trajectory.

Of all the pathological tau markers, Gallyas staining was the marker that most consistently increased in a linear fashion with age ($r^2 = 0.713$). This is the marker associated the most severe stage of tauopathy in the present study. Because NFTs are resistant to degradation, they continue to accumulate throughout the progression of pathology. In the progression of disease, the neuron eventually dies and there is evidence of a ghost tangle or the remnants of the argyrophilic tau filaments that are left behind. (Ikeda, Haga, Oyanagi, Iritani, & Kosaka, 1992).
Because the ghost tangles remain after neuronal death and retains the argyrophilic properties, it is expected that Gallyas staining would continue to increase with age.

Similar to Gallyas, the insoluble forms of phospho-tau follow a pattern with a linear increase over the lifespan. Because advanced tau formations and NFTs are resistant to degradation, they do not readily solubilize in RIPA detergent in the homogenization process. So, when the tissue homogenate is centrifuged, these formations remain in the detergent insoluble fractions. Therefore, it would be expected for the detergent insoluble fraction to display more pathological tau in a similar fashion as Gallyas staining.

When total tau was examined in the insoluble fraction, the marker appears to plateau and then decrease, possibly due to reduced production of tau secondary to neuron loss in older ages. In the soluble fraction total tau and pSer199/pSer202 tau decreased linearly which indicate that these markers could be more readily degradable forms of tau, whereas the pSer396 tau plateaus after a year of age implying some resistance to degradation. The histological markers appear to reflect some combination of the soluble and insoluble tau levels. HT7 labeled total tau increases early, but declines in later ages implying some accumulation, but a reduced rate of accumulation and possible reduction in later ages as less tau monomers are being produced. The pSer396 marker increases early and plateaus, similar to the soluble protein fraction. The AT8 labeled pSer202/pThr205-tau immunostaining resembles more of the insoluble pattern of pSer199/pSer202-tau accumulation by western analysis.

Research has indicated that pSer199 and pSer202/pThr205 epitopes are believed to be phosphorylated at an early stage of abnormal tau development, in what some consider a pre-tangle stage, while pSer396 becomes evident in middle to late stages of the disease as PHFs form
and progress to NFTs (Augustinack, Schneider, Mandelkow, & Hyman, 2002; Kimura et al., 1996; Luna-Munoz et al., 2007; Mondragón-Rodríguez et al., 2014; J. H. Su et al., 1996). Additional research has examined the progression of tauopathy in respect to age using the Braak stages (H. Braak et al., 2011). The Braak stages are a series of six stages that define increasing stages of pathology in the progression of AD tauopathy. When the epitopes of pSer202/pThr205 were examined, they peaked in the stages designated as a pre-tangle stage in middle-age, whereas the most severe stages of pathology peak at the most advanced ages. Work by Koss et al. (2016) continued an examination of tauopathy in respect to the Braak stages. Results found that the severity of epitopes pSer199/202 and pThr205-tau staining peaked at an earlier stage of pathology in comparison to the pSer396 epitope. The evidence of earlier pSer199 and/or pSer202/pThr205 severity, and the later progression of pSer396 confirms the current study’s findings of a progression of tauopathy of how some markers may be more resistant to degradation.

The second aim of this study was to examine how markers of tau-associated pathology including inflammation and neurodegeneration differ over the life course. Research shows that inflammatory factors are upregulated in the rTg4510 model (H. Wang et al., 2018; Wes et al., 2014). Our findings indicate the microglia markers continue to increase in a linear fashion, whereas astrocytes increase but tapers off just after one year of age. When examining markers of neurodegeneration, the present study found that the markers of neuronal staining by NeuN, hippocampal volume, and brain weight decline linearly which is consistent with the early studies of the model.

Aim three evaluated the relationships of tauopathy with tau-related pathology across the life course. As Gallyas staining was the marker that had a consistent increase across the
progression of pathology in the rTg4510 life course, there were strong relationships with the tau-related markers of pathology. The negative linear relationships for Gallyas staining and markers of neurodegeneration are consistent with the argument that NFTs contribute to neurodegeneration. With the apparent neuronal loss that is occurring in this model, this could be responsible for the reductions observed for total tau. The reduction in neurons would inherently reduce the amount of total tau production and could be the cause of the observed reduction of degradable forms of tau. Therefore, demonstrating the current declines in some of the soluble total tau whereas, more insoluble tau and NFTs continued to increase in the current study.

The relationships between later tau pathology (pSer396 and Gallyas staining) may be indicative of two different relationship patterns in respect to inflammatory markers. As demonstrated the microglia markers increase linearly, while astrocytes tend to taper off after a year of age. It is possible that the astrocytes are responding to injury to the neurons as PHFs grow in severity, whereas microglia may still be attempting to clear the ghost tangles. It is important to note, that markers of tauopathy and tauopathy-related markers vary over the life course. Therefore, because these markers are so intertwined with age, it is not too surprising that when age is controlled for, the relationships loose significance.

Most of the time course studies of rTg4510 line examine younger ages than the present study. The original research of the rTg4510 line by Santacruz et al. (2005) and Ramsden et al. (2005) only extended the time course studies to 10 months of age. Additional work conducted by Dickey et al. (2009) demonstrated non-linear phospho-tau accumulation in soluble protein fractions, while the insoluble fraction increased linearly up to 10 months of age. For the most part, these time points revealed linear increases in histological and insoluble protein measures. When examining the present study’s data, there are similar findings for age-dependent increases
at those time points. However, only when extending the time course longer can the impacts of reduced tau production be observed. These findings are instrumental for the examination of the progression of pathology using the rTg4510 model to identify at what ages these pathological changes occur. These additional findings are beneficial as it provides a more detailed time course examination that allow researchers to identify when in the life course would be most appropriate to address future studies such as earlier targets before the formation of NFTs and substantial neurodegeneration.

Recently the rTg4510 model has come under scrutiny from Gamache et al. (2019). This research group identified that the transgene insertion of the P301L mutation disrupts the Fgf14 and five additional genes that are believed to contribute to the observed phenotype of the rTg4510 model. Unfortunately, the present study was not able to examine the additional genes that may have been disrupted; however, the scope of this study was to examine how markers of pathology change with age.

In conclusion this study provided an extension of the age-series examinations of the rTg4510 line that demonstrated important differences that emerge later in the life course. It is evident that markers that are more implicated in progressed pathology continue to increase with age. Moreover, this study highlighted the importance of how some of the more readily degradable forms of tau decrease the over the life course. Therefore, this study identified important considerations when evaluating markers of tauopathy in the rTg4510 mouse line at later ages.
### Table 1.

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Host</th>
<th>Target</th>
<th>Source</th>
<th>Dilution</th>
<th>Methodology</th>
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<tbody>
<tr>
<td>HT7</td>
<td>Mouse</td>
<td>Total Tau</td>
<td>Invitrogen MN1000B Invitrogen MN1000</td>
<td>1:5k</td>
<td>IHC</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1:10k</td>
<td>Western Blot</td>
</tr>
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<td>AT8</td>
<td>Mouse</td>
<td>Phosphorylated tau at Serine 202 and threonine 205 epitopes</td>
<td>Invitrogen MN1020B</td>
<td>1:10k</td>
<td>IHC</td>
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<tr>
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<td>Phosphorylated tau at the Serine 396 epitope</td>
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<td>1:30k</td>
<td>IHC</td>
</tr>
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<td>Major histocompatibility complex II</td>
<td>BD Pharmingen 556999</td>
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<td>IHC</td>
</tr>
<tr>
<td>CD45</td>
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<td>Microglia</td>
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</tr>
<tr>
<td>Iba1</td>
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<td>Microglia</td>
<td>Fuji 019-19741</td>
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<td>Astrocyte</td>
<td>Dako Z0334</td>
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<tr>
<td>NeuN</td>
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<td>Neurons</td>
<td>Millipore MAB377b</td>
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<tr>
<td>PSD95</td>
<td>Rabbit</td>
<td>Presynaptic structural</td>
<td>Cell Signaling 2507S</td>
<td>1:1k</td>
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### Table 1. (Continued)

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<tr>
<th>Secondary Antibodies</th>
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<tr>
<td>Biotinylated Anti-Rabbit IgG</td>
<td>Goat</td>
<td>Conjugates to Rabbit host primary antibodies</td>
<td>Vector Laboratories BA-1000</td>
<td>1:3k</td>
<td>IHC</td>
</tr>
<tr>
<td>Biotinylated Anti-Rat IgG</td>
<td>Goat</td>
<td>Conjugates to Rat host primary antibodies</td>
<td>Vector Laboratories BA-9400</td>
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<td>IHC</td>
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<tr>
<td>680cw Anti-Rabbit IgG IR dye</td>
<td>Goat</td>
<td>Conjugates to Rabbit host primary antibodies</td>
<td>LI-COR Biosciences 926-32211</td>
<td>1:10k</td>
<td>Western Blot</td>
</tr>
<tr>
<td>800cw Anti-Mouse IgG IR dye</td>
<td>Goat</td>
<td>Conjugates to Mouse host primary antibodies</td>
<td>LI-COR Biosciences 926-32280</td>
<td>1:10k</td>
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Figure 1. Gallyas staining increases linearly with age in Tg4510 mice. Brain sections from mice ranging in age from 50 to 590 days were stained as described in methods. The average area occupied by positive staining was combined for anterior cortex, posterior cortex, and hippocampus. Results were then normalized by calculating z-scores to facilitate comparison with other markers over the lifespan. The line of fit and standard error of the mean (shaded area) for linear, quadratic, and cubic models of fit are shown.
Figure 2. Best fit equations for four histological measurements of tau deposition indicate different relationships with age. The line of fit and the standard error of the mean (shaded) are shown. Brain sections from mice ranging in age from 50 to 610 days were stained as described in methods. The average area occupied by positive staining was combined for anterior cortex, posterior cortex, and hippocampus. Results were then normalized by calculating z-scores to facilitate comparison with other markers across the lifespan.
Figure 3. Best fit equations for three measurements of soluble tau indicate different relationships with age. The line of fit and the standard error of the mean (shaded) are shown. The posterior cortex was dissected, homogenized, and the detergent soluble fraction isolated as described in methods. Tau variants were measured by immunoblot after separation of proteins by electrophoresis (western blots). Staining intensity was normalized by comparison to total protein. Results were then converted to z-scores to facilitate comparison with other markers across the lifespan.
Figure 4. Best fit equations for three measurements of detergent insoluble tau indicate different relationships with age. The line of fit and the standard error of the mean (shaded) are shown. The posterior cortex was dissected, homogenized, and the detergent insoluble fraction isolated and dissolved in formic acid as described in methods. Tau variants were measured by immunoblot after separation of proteins by electrophoresis (western blots). Immunostaining intensity was normalized to total protein staining of the gel. Results were then converted to z-scores to facilitate comparison with other markers across the lifespan.
Figure 5. Best fit equations for four histological measurements of glial activation indicate mostly linear increases with age. The line of fit and the standard error of the mean (shaded) are shown. Brain sections from mice ranging in age from 50 to 590 days were stained as described in methods. The average area occupied by positive staining was combined for anterior cortex, posterior cortex, and hippocampus. Results were then normalized by calculating z-scores to facilitate comparison with other markers across the
Figure 6. Best fit equations for four measures of neurodegeneration indicate mostly linear decreases with age. The line of fit and the standard error of the mean (shaded) are shown. Brain weights were collected at necropsy. Hippocampal volume was estimated using 8 sections through the entire region by the method of Cavalieri. Brain sections from were stained for NeuN as described in methods, and PSD-95 was measured in posterior cortical total homogenates by immunoblot. Results were then normalized by calculating z-scores to facilitate comparison with other markers across the lifespan (left axis) with the exception of the brain weight, which is shown in mg wet weight (right axis).
Table 2.

*Correlation Table of Histological Tau with Indicators of Inflammation and Neurodegenerative Markers.*

<table>
<thead>
<tr>
<th></th>
<th>CD45</th>
<th>Iba-1</th>
<th>MHCI</th>
<th>GFAP</th>
<th>Hippocampal</th>
<th>NeuN</th>
<th>Brain</th>
<th>PSD95</th>
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<tr>
<td><strong>Volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT7</td>
<td>.537**</td>
<td>.265</td>
<td>.121***</td>
<td>.433*</td>
<td>-.282</td>
<td>-.152</td>
<td>-.374*</td>
<td>-.481**</td>
</tr>
<tr>
<td>AT8</td>
<td>.481**</td>
<td>.424</td>
<td>.361</td>
<td>.514**</td>
<td>-.453**</td>
<td>-.102</td>
<td>-2.63</td>
<td>-.640***</td>
</tr>
<tr>
<td>pSer396</td>
<td>.622***</td>
<td>.657***</td>
<td>.599**</td>
<td>.751***</td>
<td>-.724***</td>
<td>-.049</td>
<td>-.327</td>
<td>-.684***</td>
</tr>
<tr>
<td>Gallyas</td>
<td><strong>.775</strong>*</td>
<td><strong>.671</strong>*</td>
<td>.583**</td>
<td>.679***</td>
<td><strong>-847</strong>*</td>
<td><strong>-.577</strong></td>
<td><strong>-.388</strong></td>
<td><strong>-.811</strong>*</td>
</tr>
</tbody>
</table>

Table 2. Pearson correlation table of four histological measurements of tau deposition indicate strong relationships with markers of inflammation and neurodegeneration. Brain sections from mice ranging in age from 50 to 610 days were stained as described in methods for NeuN, tau, and inflammatory measurements. The average area occupied by positive staining was combined for anterior cortex, posterior cortex, and hippocampus. Hippocampal volume was estimated using 8 sections through the entire region by the method of Cavalieri. Brain weight was measured in mg. PSD-95 was measured in posterior cortical total homogenates by immunoblot. Results were then normalized by calculating z-scores to facilitate comparison with other markers over the lifespan. *p < 0.05. **p < 0.01. ***p < .001. **Bolded text** indicates the strongest correlations for each histological tau marker.
CHAPTER THREE:
THE IMPACT OF MOUSE AGE UPON THE SEVERITY OF TAU PATHOLOGY
AFTER VIRAL TAU\textsuperscript{P301L} INTRACRANIAL INJECTIONS

Introduction

Tauopathies define a cluster of diseases that are defined by the presence of pathological tau, with the most common being Alzheimer’s disease (AD). Tau transgenic mouse models express an abundant amount tauopathy from early in the life course (Ramsden et al., 2005). This early expression, while helpful in animal models, may not translate well to the tauopathy found in older adult humans. Many researchers have employed the use of adeno-associated viral vectors (AAV) to produce tauopathy. This approach has been demonstrated to be a convenient means of causing tauopathy (Cook et al., 2015; Jaworski et al., 2009; X. Liu et al., 2017; Wegmann et al., 2019). Cook et al. (2015) compared the expression of viral tau\textsuperscript{P301L} following intracerebroventricular injections to the expression of the rTg4510 tau transgenic line. This study found comparable tau expression and accumulation in both models, validating the model for pathology. Furthermore, many different studies have used various AAV serotypes to model tauopathy. Cearley and Wolfe (2006) compared several serotypes and found that AAV9 was the serotype that had the largest dispersion of pathology to additional brain regions from the injection site, including the contralateral side of the brain.
Because the AAV model allows for initiation of a viral spread of pathology, this allows researchers to examine the impact of age on pathology onset. This is an important advantage because age is the greatest risk factor for an AD diagnosis and this viral model allows for the examination of age, whereas, this is not feasible in transgenic models. Wegmann et al. (2019) examined differences in tau expression using C57BL/6 mice aged between 3 and 22-24 months. After a viral incubation period of 10 weeks, it was evident that the aged mice had more tauopathy that spread to surrounding regions when compared to younger animals. Therefore, demonstrating the ability to detect differences of age in viral models of tauopathy.

During aging, there is evidence that the body’s inflammatory state becomes primed to respond to pro-inflammatory stimuli with a hyperactive release of cytokines and chemokines (Franceschi & Campisi, 2014; Franceschi et al., 2007). While there is an increase in the immune response of inflammation with age, these processes become less efficient with age (Licastro et al., 2005). The system becomes less efficient at removing pathogens and pathology accumulation and take significantly longer to recover from immune activation. Further analysis that have examined the increased inflammatory status with age in relation to tau has demonstrated this increased inflammatory status increases tau burden (Ghosh et al., 2013; Lee et al., 2010). Because of this relationship, there is interest in further understanding if this aged innate immune response accelerates tauopathy, and if so, which features of this response might be most impactful and possibly amenable to intervention. Thus, this study used intracranial AAV9 tau^{P301L} and AAV9 GFP in young, middle-aged, and old animals to establish a model to investigate tauopathy based on the age of onset and how the aging immune response may impact tauopathy in three aims.
Aim One: To evaluate the effects of the injection (AAV9 GFP or AAV9 tau\textsuperscript{P301L}) and age (young, middle-aged, and old) on markers of tauopathy.

Aim one hypotheses: All tau\textsuperscript{P301L} injected animals will have pathological tau accumulation, but with the tau\textsuperscript{P301L} injected oldest animals having the most severe pathology.

Aim Two: To evaluate the effects of the injection (AAV9 GFP or AAV9 tau\textsuperscript{P301L}) and age (young, middle-aged, and old) on markers of tau-related pathology such as immune activation and neurodegeneration.

Aim two hypotheses: Because of the increase in immune activation with age, it is hypothesized that there will be age-dependent increases in inflammation regardless of the injection group. However, the immune response will be more exaggerated in the AAV9 tau\textsuperscript{P301L} injected animals. Furthermore, there will be more atrophy and neurodegeneration observed in the tau\textsuperscript{P301L} injected animals when compared to the GFP injected animals, but this will be most severe in the oldest animals due to hypothesized increased tau burden.

Aim Three: In modeling tauopathy, it is important to have translational ability to induce cognitive deficits. Therefore, to evaluate the effects of the injection (AAV9 GFP or AAV9 tau\textsuperscript{P301L}) and age (young, middle-aged, and old), behavioral assessments were employed to determine if there was the presence of any tau related cognitive impairments.

Aim three hypotheses: The AAV9 tau\textsuperscript{P301L} injected animals will perform worse on behavioral assessments. Because it is anticipated for the old tau\textsuperscript{P301L} injected animals will have the most pathology, it is also expected for these animals to perform the worst on behavioral measures.
Method

Animals and Experimental Design

Female \((n=35)\) and male \((n=34)\) C57BL/6 mice were ordered from the National Institute of Aging. Animals were aged to 8, 12, 16 months of age and these roughly translate to young adult, middle-aged, and older adulthood (Flurkey, Currer, & Harrison, 2007). Animals were kept on a 12-hour light cycle and were provided food water *ad libitum*. Once animals were aged, stereotaxic surgeries were conducted. Prior to injections, all animals were anesthetized with 3% isoflurane (Henry Schein; Melville, NY, USF; Cat. No. 029404) and received 0.5mg/kg buprenorphine SR (ZooPharm; Laramine, WY, USA) for post-operative analgesia. 2uL of viral injections were administered to bilateral hippocampi (coordinates ML:+/-2.7, AP:-2.7, DV:-3.0) and anterior region of the cortex (coordinates ML:+/-2.20, AP:2.2, DV:-3.0) relative to bregma using a glass pulled pipette needle attached with heat shrink tubing to a 10μL Hamilton syringe (Hamilton, Pompton Plains, NJ; cat. no. 80000) at a rate of 0.5μL/minute. Weights were measured weekly to confirm the general health status of the mice post-surgery. At 3.5 months post-surgery, behavioral assessments were conducted to determine if there was any evidence of cognitive impairment. Two weeks after behavioral assessments, brain tissues were collected (Figure 7).

Viral Production

Glycerol stock of competent SURE2 cells containing green fluorescent protein (GFP) plasmid that included the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was graciously provided by Dr. Kevin Nash from the University of South Florida. WPRE can enhance expression of viral cargo genes and elongate the period of viral expression.
The tau$^{P301L}$ cDNA was obtained from Genewiz (Genewiz, South Plainfield, NJ, USA). The expression plasmids contained cytomegalovirus (CMV) enhancer with chicken β actin (CBA) promoter. This allows for expression in the CNS with limited expression in motor neurons (Gray et al., 2011). To construct the tau$^{P301L}$ plasmid, an additional GFP plasmid was cloned, and enzyme restriction digests were conducted at the AgeI and SalI sites to remove the GFP genetic sequence. Then ligation was performed to insert the tau$^{P301L}$ genetic sequence to the GFP plasmid backbone containing WPRE. Both viruses were transfected with the AAV9 plasmid and XX6 helper plasmid in HEK293T cells. Viruses were collected and purified using iodixanol gradients and ultracentrifugation. Viruses were then concentrated using Amicon ultra-15 centrifugal filters (Sigma, Burlington, MA, USA; Cat. No. UFC910024) with DPBS (pH 7.4). Ten μL aliquots of virus were stored at -80°C until the day of injection to avoid multiple freeze and thaw cycles. Viral titering was conducted using RT qPCR with primers targeted to the WPRE sequence. Both viruses were diluted to $8.8 \times 10^{11}$ vector genomes (vg)/uL. To ensure that the intracranial injections were successful and estimate the original dispersion of the tau virus, the AAV9 tau$^{P301L}$ injections also included $8.8 \times 10^{10}$ vg/uL of AAV9 GFP.

**Behavioral Assessments**

All behavioral tests were conducted by an experimenter blind to injection and age groups of all mice. Open field assessment was conducted to determine general activity and to acclimate the animals to behavioral handling. Animals were placed in the open field (L 40mm/ W 40mm/H 40mm) enclosure from BioSeb (Vitrolles France) and tracked with AnyMaze software (Stoelting, Wood Dale, IL, USA; cat. no. 60000) for 10-minute trials. The total distance traveled was measured. Rotarod was used to evaluate the animal’s motor skill and to identify any potential motor impairments. This test was conducted over two days with 4 trials on each day. Animals
were placed on the TSE (TSE Systems, Chesterfield, MO, USA) rotarod apparatus, which is a rotating rod that increases in speed through the duration of the 5-minute trial. There was at least 20 minutes between each trial to minimize muscle fatigue over trials. The latency to fall was recorded for analysis.

To evaluate visuospatial navigation memory, the radial arm water maze (RAWM) was conducted. A detailed protocol has been previously published along with the scoring sheets (Alamed, Wilcock, Diamond, Gordon, & Morgan, 2006). This test was conducted over four days in a 6-arm maze that radiates from the center that can be filled with water and external cues are placed on the wall. For each animal, the goal arm of the 6-arm maze that houses the platform remained the same, but the start arm was changed with each trial. Furthermore, the goal arm was changed between animals to avoid any overt olfactory cues. Across all four days, each animal underwent 15 trials for 1 minute each. On day one, mice were trained by alternating between a visible and hidden platform. Furthermore, if the mouse failed to reach the platform on day one after the 1-minute trial, the animal was guided to the platform. The number of incorrect arm entries that included all four limbs to be in the arm were recorded as errors. Day two involves only the hidden platform, again, incorrect arm entries were noted. Day three measured reversal learning. This involved the hidden platform being switched to the arm that is 180° across the maze from the original goal arm. Finally, on day four mice were tested for capability to see, swim towards, and ascend the platform. The visible cues were removed from the wall and the visible platform was placed in the center of the maze. No flag was used. The latency to reach the visual platform was recorded. The number of errors and platform latency were averaged in sets of three to create 5 blocks of trials for each day.
Tissue Collection

Four months after the injection of the AAV viruses, tissues were collected in the same manner as the previous study. The mice were anesthetized with 0.1mL/10g Somnasol (0.25mL in 10mL of H₂O) and placed on a heating pad to maintain body heat to reduce any artefactual tau phosphorylation caused by reduced body temperature during anesthesia (Planel et al., 2007). Animals were perfused transcardially with 25mL of 0.9% normal saline solution. Once the animals were perfused, the brains were immediately extracted. The brains were placed on an iced stage and the right hemisphere was dissected into the anterior region of the cortex (ACX), posterior region of the cortex (PCX), and the hippocampus (HPC) and brain regions were immediately frozen on dry ice. Brain region specimens were stored at -80°C until homogenization for biochemical analysis. The left hemisphere was immediately immersed in 20mL of freshly prepared 4% paraformaldehyde (pH 7.4) for 24 hours at 4°C. After 24 hours, the left hemispheres were cryoprotected, where each brain was placed successively in 10, 20, and 30% sucrose for 24 hours. The brain hemispheres were stored at 4°C until sectioned. Brains were horizontally sectioned with a freezing microtome at a 25μm thickness and placed into Dulbecco’s phosphate buffered saline with 10 mM sodium azide solution (7.4 pH) except for every 10th section that was sectioned at 50μm thickness for hippocampal volumetric analysis.

Tissue Homogenization and Biochemical Analysis

The HPC was added to 10v/w to RIPA (50mM Tris-Cl, 150nM NaCl, 1% NP40, 0.1% SDS, 0.05% sodium deoxycholate) buffer with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P8340), deacetylase inhibitor cocktail (MedChem Express, Monmouth Junction, NJ, USA; Cat. No. HY-K0030) phenylmethanesulfonyl fluoride (Sigma
Aldrich, St. Louis, MO, USA; Cat. No. P7626), and phosphate inhibitor cocktails II and III (Sigma Aldrich, St. Louis, MO, USA; Cat. Nos. P5726 & P0044, respectively).

Samples were then homogenized first by a rotating mechanical pestle and then by sonication. Once sonicated, a consistent aliquot was removed from each sample and then centrifuged at 50,000g for 1 hour at 4°C. The remaining crude homogenate was stored at -80 °C until analysis. Once the centrifugation was complete, the supernatant was removed and stored as the detergent soluble fraction, and the detergent insoluble pellet was kept for formic acid processing. Formic acid processing was conducted by adding a fifth of the total sample volume of 70% formic acid to each pellet and homogenized by a rotating mechanical pestle. After the pellets were homogenized, the samples were gently rotated for 30 minutes. 15.1M NaOH was added to neutralize formic acid samples. Once neutralized, 1M Tris-Base (pH 7.6) was added to a 1:2 ratio to the homogenate volume. Individual insoluble samples were adjusted to pH of 7. Protein concentrations for crude, supernatant, and formic acid fractions were determined by Pierce BCA protein assays.

Samples for both the detergent soluble and insoluble fractions were used for all tau ELISA assays. The assays were used to detect total tau (Fisher Scientific, Cat. No. KHB0041), phosphorylated Ser199-tau (Fisher Scientific, Cat. No. KHB7041), and phosphorylated Ser396-tau (Fisher Scientific, Cat. No. KHB7031). All samples were first diluted (1:100) in RIPA, then diluted appropriately for each ELISA assay in the provided ELISA kit’s buffer. All concentrations derived from the ELISA assay were normalized to the sample’s total protein concentration.
**Histopathology**

Tissues from both AAV9 GFP and AAV9 tau\textsuperscript{P301L} groups were placed in free-floating multi-sample staining trays for each marker. The trays were placed in an endogenous peroxidase blocking solution (10% methanol, 3% H\textsubscript{2}O\textsubscript{2} in PBS). The tissues were washed 3 times with PBS and then placed in permeabilizing solution (0.2% lysine, 1% Triton X100, 4% normal goat serum in PBS). Tissues were then incubated overnight in the appropriate antibody (Table 3). The following morning, tissues were washed with PBS and incubated for 2 hours in the corresponding biotinylated secondary antibody. This step was excluded if the primary antibody was biotinylated. After the two-hour incubation period, tissues were washed with PBS and placed in Vectastain\textsuperscript{®} Elite\textsuperscript{®} ABC kit solution (Vector Laboratories, Burlingame, CA; cat. no. PK-6100) to enhance the enzyme complex. Again, tissues were washed with PBS for 2 washes and TBS for a final wash. Following the TBS wash, tissues were placed in a color development solution (0.05% DAB, 0.5% Ni\textsuperscript{2+}, .03% H\textsubscript{2}O\textsubscript{2},) for 5 minutes. To stop color development, tissues were washed with TBS for one wash and then 2 PBS washes. Upon the completion of staining, tissues were mounted on microscope slides (Fisher Scientific, Waltham, MA; cat. no. 1255015), dehydrated, and cover slipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA; cat. no. 13512).

For Gallyas and cresyl violet staining, tissue sections were first mounted on microscope slides and dried overnight. Gallyas staining is a silver stain that identifies NFTs (Uchihara, 2007). On the day of Gallyas staining, sections were briefly rehydrated and then placed in a pretreatment solution (5% periodic acid). After a series of washes, the slides were immersed in a silver enhancing solution (10% potassium iodide, 4% NaOH, 0.035% silver nitrate) for one minute before incubating for 10 minutes in a 0.5% acetic acid solution. Slides were then placed
in a working developer solution (2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.5% tungstosilic acid, 0.2% formaldehyde) for approximately 10 minutes. Upon development, slides were washed with 0.5% acetic acid followed by water washes. Then slides were immersed in a gold toning solution (0.1% gold chloride solution) followed by an additional water wash. Then slides were incubated in a solution to remove traces of unconjugated silver salts (0.1% sodium thiosulphate solution) and a final water wash. Slides were immediately dehydrated and coverslipped.

The cresyl violet stain was conducted on pre-mounted 50μm tissues. The slides were briefly rehydrated and then placed in a 0.05% cresyl violet dye solution. After staining, slides were briefly placed in an acetic acid water solution (pH 3.5) and rinsed in water. Slides were then dehydrated and coverslipped in DPX (Electronic Microscopy Sciences Cat. No. 13512). Once the DPX was dried, slides were scanned using ZEISS Axio Scan.Z1. Scanned slides were analyzed using NearCyte tissue analysis IAE software (created by Andrew Lesniak). The entire tissues section was identified and immunopositive pixels above a user-defined threshold were measured. The threshold parameters were derived using several sections with light and dark staining and held constant throughout the analysis. The fraction of the total region occupied by immunopositive pixels was calculated. Cresyl violet staining was conducted to measure hippocampal volume using the method of Cavalieri.

**Statistical Analysis**

All statistical analyses were conducted using IBM SPSS Statistics 25 (Armonk, New York, USA) and figures were created with GraphPad Prism 8 (La Jolla, CA). For aim one of this study to address any group differences on markers of tauopathy from histological staining and
ELISA assay concentrations based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged, and old), two-way ANOVAs were conducted. To address aim two, to determine if there were any group differences based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged, and old) for histological markers of tau-related pathology of inflammation and neurodegeneration, two-way ANOVAs were also conducted. For aim three, to assess any evidence of group differences based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged) in the behavioral assessment, a two-way ANOVA or two-way repeated measures ANOVAs were conducted. For the first behavioral task, the open field examined the total distance traveled in a ten-minute trial, so a two-way ANOVA was conducted. However, the rotarod and RAWM are tasks that have multiple trials; therefore, two-way repeated measures ANOVAs were conducted. To assess motor function, the rotarod was used to measure the time each mouse spent on a rotating rod during eight five-minute trails over the course of two consecutive days. A two-way repeated measures ANOVA was conducted to identify any group differences based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged, and old). Finally, for each day of the RAWM, repeated measures two-way ANOVAs were conducted to determine any group differences based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged, and old) on the average of errors for five blocks of trials for day one, day two, and the reversal task. For the open pool task, time to reach the platform was analyzed by a two-way repeated measures ANOVA to determine any group differences based on injection (GFP vs tau\textsuperscript{P301L}) or age (young, middle-aged, and old). All significant ANOVAs were followed by Fisher’s least significance difference (LSD) post-hoc analyses to identify the groups that were significantly different.
Results

**Aim One: Effects of Age Differences on Tauopathy**

*GFP and total tau expression*

The overarching goal of this study was to examine if the age of animal at the onset of a viral method of inducing tauopathy could impact the severity of pathology. To first determine that the intracranial injections were successful, immunostaining for GFP and total tau expression were conducted. Histological GFP and tau representative images can be seen in Figure 8. For most injections in the hippocampus, the GFP immunoreactivity filled most of the structure in both the GFP group and in the P301L tau groups, that also included a 1/10th dose of the GFP virus in addition to the tau virus. The GFP staining area was measured using image analysis and a two-way ANOVA was conducted to determine group differences based on age and injection for histological GFP. There was a main effect of injection group, $F(1,58) = 495.21, p < .001$, where the GFP injected animals had significantly more GFP staining when compared to the tau$^{P301L}$ injected animals (Figure 9A). The age groups did not have any significant differences in GFP staining ($p = .478$). As expected, when examining histological total tau measured by HT7, there was no detectable staining in mice injected with the GFP virus, resulting in a significant main effect of injection, ($F(1,58) = 194.43, p < .001$; Figure 9B). Also, HT7 staining did not vary significantly when age groups were examined ($p = .599$). After measuring total tau concentration by ELISA assays using both the detergent soluble and detergent insoluble fractions, two-way ANOVAs were conducted. There were main effects of the viral injection group for both detergent soluble, ($F(1,58) = 135.42, p < .001$, Figure 9C) and detergent insoluble ($F(1,55) = 52.46, p < .001$, Figure 9D) fractions, where there was evidence of total tau in the
tau$_{\text{P301L}}$ injected animals and no total tau in the GFP injected animals. Similar to histological total tau, both soluble ($p = .393$) and insoluble ($p = .753$) total tau did not change statistically significantly with age. There were three animals that did not have enough insoluble sample to run in the ELISA assays that included one young tau$_{\text{P301L}}$ injected, middle-aged tau$_{\text{P301L}}$ injected, and one old GFP injected mouse. These three animals were not included for any of the detergent insoluble analyses.

*Phospho-tau epitopes*

To further evaluate tauopathy, phospho-tau isoforms were measured both histologically (Figure 8) and by ELISA assays (Figure 10). Two-way ANOVAs were conducted to determine the effect of injection group and age at onset of pathology to determine group differences for indicators of phosphorylated tau. Histological phospho-tau Ser202/Thr205, measured by AT8, not only had main effects of both injection group ($F(2,57) = 93.17, p < .001$) and age ($F(2, 57) = 3.61, p = .033$), but the interaction of age and injection ($F(2,57) = 3.42, p = .040$) was significant (Figure 10A). There was no AT8 staining in the GFP injected animals. For the tau$_{\text{P301L}}$ injected animals, Fisher’s LSD post-hoc analysis indicated that the old animals ($M = .050, SEM = .004$) displayed significantly more AT8 staining when compared to the young animals ($M = .025, SEM = .005; p = .032$). After measuring the concentration of phospho-tau Ser199 (pSer199) in the detergent soluble and detergent insoluble fractions, two-way ANOVAs were conducted to determine differences between injection group and age. For both the detergent soluble ($F(1,58) = 78.33, p < .001$) and detergent insoluble pSer199-tau ($F(1,55) = 38.92, p < .001$) there were main effects of injection group, where the tau$_{\text{P301L}}$ injected animals demonstrated the presence of pSer199 and the GFP inject animals did not (Figure 10B-C). However, both fractions failed to have any significant effects of age ($p > .514$).
To examine group differences based on injection and age group on indicators of late-stage tau phosphorylation, two-way ANOVAs were conducted for indicators of phospho-tau Ser396 (pSer396). Histological pSer396-tau had a main effect of injection group \((F(1,58) = 68.273, p < .001)\), but did not have any main effect of age (Figure 10D). The tau^{\text{P301L}} injected animals had evidence of pSer396 staining, but GFP injected animals did not. When examining the soluble fraction pSer396-tau concentration, there was also a main effect of injection \((F(1,58) = 39.719, p < .001)\) where the tau^{\text{P301L}} injected animals demonstrated pSer396-tau compared to no pSer396 detected in the GFP injected animals (Figure 10E). There was no evidence of an age effect \((p = .890)\). Consistent with the soluble pSer396, the insoluble fraction had a main effect of injection, \((F(1,55) = 27.07, p < .001)\), but failed to detect a main effect for age \((p = .418; \text{Figure 10F})\). Still, although not statistically significant, there was a trend for both the histological measure of pSer396-tau and ELISA measured insoluble pSer396-tau to increase in older mice.

*Neurofibrillary tangles*

To further estimated the severity of tauopathy silver staining of tau fibrils (NFTs) using the Gallyas method was conducted and analyzed by two-way ANOVA. Main effects of injection \((F(1, 58) = 87.03, p < .001)\) and age \((F(2,58) = 6.66, p = .002)\) were significant. The tau^{\text{P301L}} injected animals had evidence of Gallyas staining whereas, there was no evidence of staining in the GFP injected mice. In addition, there was evidence of an age-dependent increase in Gallyas staining for the tau^{\text{P301L}} injected animals, evidenced by a significant interaction of age and injection \((F(2,58) = 7.29, p = .001; \text{Figure 11})\). Fisher’s LSD post-hoc analysis confirmed that the old mice had significantly more Gallyas staining when compared to young \((p = .004)\) or middle-age \((p = .015)\) mice.
Aim Two: Effects of Injection and Age on Immune Activation and Neurodegeneration

Microglia

In the aging process there is a general increase of immune activation that includes markers of microglial activation. The current study posited that the aging immune system could impact the rate of tauopathy. A two-way ANOVA was conducted to determine if there were group differences of injection and age on microglia measured by Iba-1. There was no significant difference based on injection group \( (F(1,58) = 1.06, p = .307) \) or age \( (F(2,58) = 0.63, p = .534) \) for Iba-1 (Figure 12A). In addition, microglia activation can induce MHCII expression, so staining by MHCII was conducted (Figure 12B). There were significant effects for injection \( (F(1,58) = 13.76, p < .001) \) and age \( (F(2,58) = 4.76, p = .012) \). These results indicate that there was more MHCII expression in the tau\(^{p301L}\) injected mice \( (M = 0.041, SEM = 0.004) \) when compared to the GFP injected mice \( (M = 0.022, SEM = 0.004; \) Figure 12B). Fisher’s LSD post-hoc analysis determined the young animals \( (M = 0.020, SEM = .005) \) had significantly less MHCII expression when compared to middle aged animals \( (M = 0.036, SEM = .005; p = .027) \) and old animals \( (M = 0.039, SEM = 0.004; p = .006) \).

Neurodegeneration

A neuronal stain, NeuN, was used to determine if there was any effect of injection or age on neurons. A two-way ANOVA failed to find a significant effect of injection group \( (F(1,58) = 2.18, p = .146; \) Figure 13A). There was also no significant effect of age \( (F(2,58) = 1.20, p = .380) \), although there was a trend for less NeuN staining in the oldest mice injected with tau virus. In addition, hippocampal volume was assessed by Nissl staining and using the method of
Cavalieri to estimate the volume. A two-way ANOVA indicated there was a significant reduction of hippocampal volume in the tau$^{P301L}$ injected animals when compared to the GFP injected animals, $(F(1,58) = 18.178, p < .001$, See Figure 13B), but there was no significant effect of age $(p = .558)$.

**Aim Three: Effects of Injection and Age on Cognitive Measures**

*Open field*

The first behavioral measure was the open field to assess general activity. The total distance traveled over a 10-minute trial was measured and a two-way ANOVA was conducted to identify group differences of injection and age. Results found a significant main effect of age $(F(2,58) = 5.46, p = .007$; Figure 14). Fisher’s LSD post-hoc analysis found that old animals $(M = 29.67, SEM = 2.943)$, irrespective of injection group, traveled significantly less distance when compared to young $(M = 40.70, SEM = 3.377; p = .016)$ and middle-aged animals $(M = 43.17, SEM = 2.943; p = .004)$.

*Rotarod*

Mice were tested for locomotor activity by their capacity to remain on an accelerating rotating rod. A two-way repeated measures ANOVA was conducted combining the two days of testing for the rotarod (Figure 15). Results were consistent with the open field, where there was a significant effect of age $(F(2,58) = 13.74, p < .001)$, but not injection group. Fisher’s LSD post-hoc identified an age-dependent effect where the young animals spent the most time on the rotarod when compared to middle-age $(p = .049)$ and old $(p < .001)$ animals (Figure 15B and C). Additionally, the middle-age animals spent significantly more time on the rotarod when
compared to old \((p = .003)\) animals. Within-subjects tests indicate \((F(5.67, 328.57) = 25.66, p < .001)\) that all groups improved on the task over the two days of testing.

*Radial arm water maze*

To examine any translational effect of cognitive impairment, repeated measures two-way ANOVAs were conducted on the radial arm water maze tasks to determine any effects of injection group or age (see Figure 16). On day one, the training day, there was no significant effect of injection \((F(1,33) = 0.024, p = .879)\) or age \((F(2,33) = 0.225, p = .8800)\). On day two of the RAWM, or the testing day, there was a significant main effect of age \((F(2, 33) = 7.38, p = .002)\) and a significant interaction of age and injection group \((F(2,33) = 3.61, p = .038)\). Fisher’s LSD post-hoc analysis indicated that older mice made significantly more errors when compared to young \((p = .001)\) and middle-aged \((p = .021)\) mice. However, when examining the interaction of age and injection, the old GFP injected animals \((M = 4.17, SEM = .527)\) performed more errors on the second day of the RAWM when compared to young \((M = 1.16, SEM = .416)\) middle-aged \((M = 1.83, SEM = .445)\) GFP injected animals, whereas for the tau\(^{P301L}\) injected animals, the three age groups performed similarly with no age differences (Figure 17).

To assess the capacity to learn a new platform location, the location of the hidden platform arm was reversed on the third day of RAWM testing. A two-way repeated measures ANOVA did not find any significant differences based on injection group \((F(1,33) = 1.96, p = .170)\) or age \((F(2,33) = .544, p = .585)\) (Figure 18A). To ensure that the animals were able to reach and ascend the platform without visual impairment, the open pool task with a visible platform was conducted. If any animal was unable to complete the task in 20 seconds or less on the final trial, the animal was excluded from all RAWM tasks due to potential visual impairment.
(Figure 18B). A two-way repeated measures ANOVA revealed no group differences on the animals that were able to reach the platform within 20 seconds based on injection \((F(1,33) = 0.200, p = .658)\) or age \((F(2,33) = 0.022, p = .978)\).

There were 5 animals that died prior to behavioral assessments. Deaths were attributed to old age or other inexplicable causes. However, it should be noted that there were 22 additional animals excluded from RAWM analyses. Of these 22 animals, 12 animals were not able to swim over the four days of testing in the water maze, 6 had visible eye problems, and 4 had seizures on days of testing. The oldest animals were the ones most afflicted by health concerns (50%) and inability to swim (83.3%). Three animals had to be excluded from all RAWM tasks due to not reaching the visible platform within 20 seconds on the last trial of the open pool task. The final sample sizes in each group were young GFP \(n = 8\), middle-aged GFP \(n = 7\), old GFP \(n = 5\), young tau \(n = 10\), middle-aged tau \(n = 5\), and old tau \(n = 4\) for the radial arm water maze analyses.

**Discussion**

The goal of this study was to examine the effects of age on the viral onset of a model of tauopathy. There were three specific aims that this study was designed to address. The first aim was to examine injection and age differences on markers of tauopathy. Total tau and GFP immunostaining indicated that the intracranial injections were successful. Tau was only expressed in mice receiving viral administration of tau\(^{P301L}\). GFP was expressed at higher levels in AAV9 GFP injected mice and filled the target area well. Because AAV9 GFP was included in the AAV9 tau\(^{P301L}\) injectate, tau injected mice also expressed GFP, but in lower levels than the AAV9 GFP injected mice. Furthermore, when age differences were examined, there were no
differences in viral expression with age, thus indicating there was no effect of age on the GFP’s or tau\textsuperscript{P301L}’s abilities to transduce neurons.

When examining markers of tauopathy, the AAV9 tau\textsuperscript{P301L} virus was able to induce pathological changes in tau similar to other viral models of tau\textsuperscript{P301L} (Jaworski et al., 2009; Klein et al., 2004; You et al., 2019). There was evidence of an age effect in the AAV9 tau\textsuperscript{P301L} for histological early phosphorylated tau and NFTs, where older animals had significantly more pathology than the younger aged animals. The present study was able to validate the ability of AAV9 tau\textsuperscript{P301L} to produce a hallmark indicator of AD-related pathology, Gallyas positive NFTs. In addition, the detergent insoluble fraction of brain homogenate samples had evidence of late phosphorylated tau (pSer396-tau) where there was a trend that the older animals had a greater concentration when compared to younger groups. However, there is a large amount of variation for the insoluble pSer396 and could be due to possible inter-animal variation where not every animal produces pathology at the same rate. This becomes more evident in the insoluble homogenate fractions and soluble and histological late phosphorylated tau. This variation could be impacted by several factors including variation in the seeding ability of tau to propagate pathology.

The second aim of this study was to examine injection and age differences on markers of immune activation and neurodegeneration. There was a clear effect of aging for MHCII staining where the young animals had significantly less expression when compared to middle-aged and old animals, where this was more exaggerated in the tau\textsuperscript{P301L} injected animals, providing evidence of this model to exhibit the increase in inflammation in the aging process. Our findings of age differences for reactive immune function, is consistent with work conducted by Cook et al. (2015) that have observed significant age effects of reactive immune function in a viral model.
of tau$^{\text{P301L}}$. However, there were no significant difference in the present study for microglia as measured by Iba-1. It is important to note that Iba-1 is a pan microglia marker, and it does not reflect only activated microglia. Additional testing of reactive inflammation should be examined for further investigation of the immune response including markers of reactive microglia such as CD11b, and CD45.

The tau$^{\text{P301L}}$-injected animals had a reduction in hippocampal volume; however, this was not impacted by age. With the observed age-dependent increase in Gallyas positive NFTs, there was an anticipation for the old tau$^{\text{P301L}}$ mice to have more atrophy when compared to the young and middle-aged tau$^{\text{P301L}}$ injected groups. Yet the amounts of atrophy were similar at all ages for tau$^{\text{P301L}}$ mice. Further investigations will be aimed at understanding potential causes for this. One avenue that warrants investigation is to determine if there is a difference in the content of tau oligomers. Tau oligomers are small hyperphosphorylated tau aggregates that some researchers suggest to be one of the most toxic forms of tau (Shafiei, Guerrero-Muñoz, & Castillo-Carranza, 2017). This toxic species occurs early in the progression of pathology; therefore, it would be advantageous to examine this factor in relation to hippocampal volume (Lasagna-Reeves et al., 2012; Maeda et al., 2006). In addition, when examining a neuronal marker, there were no significant differences, yet, it is important to note that the old tau$^{\text{P301L}}$ injected animals appeared to have a reduction in neuronal staining when compared to the young and middle-aged tau$^{\text{P301L}}$ injected animals although it failed to reach significance.

The third and final aim of this study was to address age differences in GFP and tau$^{\text{P301L}}$ injected groups on measures of motor function and visuospatial memory. The behavioral assessment indicated a significant effect of age on motor function. This effect was demonstrated on each of the tasks as the aged mice performed worse on each of the measures the open field
and rotarod. Furthermore, the aged animals were much more likely to be excluded due to an inability to swim in the memory task. The traditional criterion of successful learning in the RAWM is less than one error on the last block of trials on day two. The only groups that were able to successfully achieve this goal were the young and middle-aged GFP injected mice.

In conclusion, the AAV9 tau$^{P301L}$ virus was able to induce a model of tauopathy that exhibits an effect of age. This model included markers of tauopathy, inflammation, neurodegeneration, and behavioral deficits. The effects of age were demonstrated on early histological phospho-tau, NFTs, and activated inflammation. The behavioral measures demonstrated effects of age, where animals regardless of injection group, demonstrated worse motor performance. The visuospatial task demonstrated impairment in the each of tau$^{P301L}$ injected groups, and the old GFP group. While further investigation is warranted to understand the age differences in detail, this preliminary work has provided the necessary validation for continuing the use of this model. With further evidence of this model to demonstrate a viral model of tauopathy that can be influenced by the aging immune system, could produce a translationally meaningful representation of clinical cases. Not only would this produce a representative animal model, but it has the potential to aid in the identification of immune pathways that might be amendable to clinical intervention to reduce tau burden in the future.
Figure 7. Experimental design and flowchart with sample sizes for the examination of mouse age upon the severity of tau pathology after viral tau $\text{P301L}$ intracranial injections. Red boxes indicate attrition due to mortality, health concerns, or inability to swim.
Table 3.

Summary of Primary and Secondary Antibodies Used for Immunohistochemistry

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<th>Target</th>
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<td>Total Tau</td>
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<td>1:5k</td>
</tr>
<tr>
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<td>Mouse</td>
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<td>Invitrogen MN1020B</td>
<td>1:10k</td>
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<tr>
<td>pSer396</td>
<td>Rabbit</td>
<td>Phosphorylated tau at the Serine 396 epitope</td>
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<td>1:30k</td>
</tr>
<tr>
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<td>Major histocompatibility complex II</td>
<td>BD Pharmingen 556999</td>
<td>1:5k</td>
</tr>
<tr>
<td>Iba1</td>
<td>Rabbit</td>
<td>Microglia</td>
<td>Fuji 019-19741</td>
<td>1:5k</td>
</tr>
<tr>
<td>NeuN</td>
<td>Mouse</td>
<td>Neurons</td>
<td>Millipore MAB377b</td>
<td>1:30k</td>
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</table>

<table>
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<th>Host</th>
<th>Target</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
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<td>Goat</td>
<td>Conjugates to Rabbit host primary antibodies</td>
<td>Vector Laboratories BA-1000</td>
<td>1:3k</td>
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<td>Biotinylated Anti-Rat IgG</td>
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<td>Conjugates to Rat host primary antibodies</td>
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<td>1:3k</td>
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<td>Biotinylated Anti-Chicken IgY</td>
<td>Goat</td>
<td>Conjugates to Chicken host primary antibodies</td>
<td>Vector Laboratories BA-9010</td>
<td>1:3k</td>
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Figure 8. Micrograph representation of the hippocampal area for histological GFP and tau staining. A-D, GFP staining; E-H, total tau measured by HT7; I-L phosphorylated tau at ser202/thr205 (AT8); M-P, phosphorylated tau at ser396 (pSer396); Q-T, Gallyas staining. 16-month-old GFP injected mouse A,E, I, M,Q; B,F,J,N,R tau^{P301L} injected 12-month-old mouse; C,G,K,O,Stau^{P301L} injected 16-month-old mouse; D,H,L,P,T tau^{P301L} injected 20-month-old mouse. Scale bar 500μm.
Figure 9. Expression of GFP and tau after intracranial injections of AAV9 GFP and AAV9 tau\textsuperscript{P301L} in young, middle-aged, and old mice. (A) Quantification of the histological positive fractional area for GFP staining, $n = 64$. (B) Quantification of histological positive fractional area for total tau (HT7) staining, $n = 64$. (C) The hippocampus was dissected, homogenized, and the detergent soluble fraction was isolated, and total tau ELISA assays were used to quantify soluble total tau concentration that is displayed normalized to the total protein values, $n = 64$. (D) The hippocampal detergent insoluble fraction was isolated and used total tau ELISA assays to quantify viral expression normalized to the total protein values, $n = 61$. Data are presented as mean ±SEM (error bars). Each dot represents one mouse. ***$p < .001$. 

\textsuperscript{P301L}
Figure 10. Age-dependent increases in early histological phospho-tau pSer202/pThr205 in AAV9 tau P301L injected animals, n = 63. (A) Quantification of the histological positive fractional area of phospho-tau pSer202/pThr205 (AT8) demonstrating an age-dependent increase in the tau P301L injected animals, n = 63. (B) The pSer199 concentration measured by ELISA assays on the detergent soluble hippocampal fraction normalized to total protein, n = 64. (C) The pSer199 concentration measured by ELISA assays on the detergent insoluble hippocampal fraction normalized to total protein, n = 61. (D) Quantification of the histological positive fractional area of phospho-tau Ser396, n = 64. (E) The pSer396 concentration measured by ELISA assays on the detergent soluble hippocampal fraction normalized to total protein, n = 64. (F) The pSer396 concentration measured by ELISA assays on the detergent insoluble hippocampal fraction normalized to total protein, n = 61. Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** p < .001 * p < .05.
Figure 11. Age-dependent increase in Gallyas staining in the tau \textsuperscript{P301L} injected mice. Quantification of the histological positive fractional area for Gallyas staining by injection and age groups, \(n = 64\). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \(p < .001\). * \(p < .05\) Fisher’s LSD post-hoc analysis.
Figure 12. Quantification of histological immune markers by age and injection. (A) Quantification of histological positive fractional area ratio of pan microglia by Iba1 staining, \( n = 64 \). (B) Quantification of histological positive area ratio of MHCII expression microglia by MHCII staining, \( n = 64 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \( p < .001 \), ** \( p < .01 \) and * \( p < .05 \) Fisher’s LSD post-hoc analysis.
Figure 13. The AAV9 tau $^{\text{P301L}}$ injected mice experienced a reduction in hippocampal volume, but no significant reduction in neurons. (A) Quantification of histological positive fractional area of neurons, measured by NeuN staining, $n = 64$. (B) Quantification of hippocampal volume estimated using 8 sections through the entire region by the method of Cavalieri indicated that the tau $^{\text{P301L}}$ mice had a reduction of hippocampal volume compared to the AAV9 GFP injected mice; $n = 64$. Data are presented as mean ± SEM (error bars). Each dot represents one mouse. *** denotes $p < .001$ main effects of injection from a two-way ANOVA.
The old animals, regardless of injection group, traveled significantly less distance when compared to middle-aged and young animals, $n = 64$. Data are presented as mean ±SEM (error bars). Each dot represents one mouse. ** $p < .01$ * $p < .05$. 

Figure 14.
Figure 15. Older mice for both AAV9 GFP and AAV9 tau \(^{P301L}\) injection groups spent significantly less time on the rotarod when compared to the middle-aged and young mice. (A), Two-day rotarod performance for young, middle-aged, and old mice for both AAV9 GFP and AAV9 tau \(^{P301L}\) injected mice demonstrate an effect of age where older animals perform worse when compared to young and middle-aged mice. B, Two-day rotarod performance for young, middle-aged, and old AAV9 GFP injected mice demonstrate an age dependent decrease in performance where the increasing age groups performed significantly worse, \(n = 32\). C, Two-day rotarod performance for young, middle-aged, and old AAV9 tau \(^{P301L}\) injected mice demonstrate the old mice spent significantly less time on the rotarod when compared to young and middle-aged animals, \(n = 32\). Data are presented as mean ±SEM (error bars). *** \(p < .001\), * \(p < .05\). Fisher’s post-hoc analysis.
Figure 16. Mice did not vary by injection or age group for the average number of errors on day one of the radial arm water maze. (A) The five blocks of trials for day one of the radial arm water maze for all injection and age groups, $n = 39$. (B) The five blocks of trials for young, middle-aged, and old AAV9 GFP injected mice on day one of the radial arm water maze, $n = 20$. (C) The five blocks of trials for young, middle-aged, and old AAV9 tau $^\text{P301L}$ injected mice on day one of the radial arm water maze, $n = 19$. Data are presented as mean ±SEM (error bars). RAWM = radial arm water maze.
Figure 17. Old animals that received AAV9 GFP and AAV9 tau\textsuperscript{P301L} injections performed more errors on day two of testing for the radial arm water maze when compared to the young animals. (A) The five blocks of trials for all injection and age groups on day two of the radial arm water maze, \( n = 39 \). (B) The five blocks of trials for young, middle-aged, and old AAV9 GFP injected mice on day one of the radial arm water maze. Old animals made significantly more errors when compared to young and middle-aged animals, \( n = 20 \). (C) The five blocks of trials for young, middle-aged, and old AAV9 tau\textsuperscript{P301L} injected mice on day two of the radial arm water maze, \( n = 19 \). Data are presented as mean ±SEM (error bars). RAWM = radial arm water * \( p < .05 \) Fisher’s LSD post-hoc analysis.
Figure 18. No difference between age or injection groups for radial arm water maze reversal task or time to reach the platform on the open pool. (A) The number of errors made on the reversal task of the radial arm water maze for all injection and age groups, \( n = 39 \). (B) Time to reach the platform in the open pool task to ensure animals are free of visual impairments for all injection and age groups, \( n = 39 \). RAWM = radial arm water maze.
CHAPTER FOUR:

COMPARISON OF THREE DIFFERENT TAU VIRAL ISOFORMS

Introduction

Tau has proven to be a critical feature of AD-related pathology. It is a defining disease hallmark that is strongly related to cognitive dysfunction and neurodegeneration (Abbott et al., 2006; Bejanin et al., 2017; Gordon et al., 2018; Hanseeuw et al., 2019; Malpas et al., 2020). This has caused many researchers to focus their efforts to further understanding this protein. Currently, there is no known mutation for the tau MAPT gene that is exclusive to AD. This causes an issue for researchers as animal models are not able to directly translate the inherent tau AD-related pathology. However, other mutations related to tau have been identified and allowed for the generation of tau transgenic animal models that allow researchers to understand key biological mechanisms that occur in the progression of tauopathy. Tauopathies all include pathological tau; however, the most striking differences are the brain regions that are afflicted. The different brain regions and cell-types that are afflicted by tau burden are responsible for the clinical manifestations of the disease. For instance, AD-related tauopathy begins in the transentorhinal cortex and hippocampal regions that are associated with memory, whereas, frontotemporal dementia begins in areas of the frontal cortex related to reasoning (H. Braak & Braak, 1995; Rosen et al., 2002). Other tauopathies are associated with aberrant tau accumulation in other brain regions (corticobasal syndrome) and even glial expression (progressive supranuclear palsy). These tau aggregates appear unique because transfer of tau
aggregates associated with different diseases was maintained upon serial transfer into mice (Narasimhan et al., 2017).

AAV modeling allows researchers to specify the location of pathology (Daya & Berns, 2008). Specifically, the use of AAV serotype 9 efficiently transduces neurons within the central nervous system and when using tau constructs has proven to promote tauopathy in the central nervous system (Cearley & Wolfe, 2006; Cook et al., 2015). Because early AD-related tauopathy is apparent in the entorhinal cortex and hippocampus, AAV9 allows viral integrations to start in these critical regions. Therefore, while tau mutations are not yet identified with AD, the use of AAV9 methodology to promote tauopathy in critical AD-related areas, allows for a new models of tauopathy that might be more translational for AD. Researchers have employed AAV9 to introduce wild-type and mutated tau to induce models of tauopathy.

Wild-type tau refers to tau that lacks any mutation. These models include inducing a larger amount of tau in the brain. Because wild-type tau does not aggregate as avidly as mutant tau variants, the rate of pathology and the severity is decreased when compared to mutated tau models (Hasegawa, Smith, & Goedert, 1998; Nacharaju et al., 1999). A commonly used tau mutation in viral models is P301L. The P301L mutation refers to the substitution of proline for a leucine amino acid at the 301st residue (using the 2N4R sequence) in exon 10 located in the microtubule binding domain. This mutation has commonly been used for modeling tauopathy including the JNPL3 and rTg4510 transgenic mice (Lewis et al., 2000; Santacruz et al., 2005). These animal models exhibit extensive tau burden, inflammatory response, neurodegeneration, and deficits in cognitive function. This mutation has long been associated with increases in toxic tau forms and reduced microtubule binding (Barghorn et al., 2000; Bunker, Kamath, Wilson, Jordan, & Feinstein, 2006; Chang, Kim, Yin, Nagaraja, & Kuret, 2008).
Clinical manifestations of the P301L mutation in fronto-temporal lobe dementia include a younger mean age of symptom onset (45-64 years of age) when compared to other mutations and a disease duration between 4 and 16 years. Clinical symptoms include disinhibition including aggressive behavior, restlessness, word finding difficulties, and egocentrism (Heutink et al., 1997; Reed et al., 1997; Van Swieten et al., 1999). In a clinical study that examined three families known to have this mutation, individuals showed evidence of neuronal loss and cortical atrophy coupled with evidence of inflammation (Heutink et al., 1997; Van Swieten et al., 1999).

R406W is a mutation that is the result of an arginine amino acid being replaced by tryptophan and is located on exon 13 outside of the microtubule binding domain. While this mutation has not been used for viral applications, the mutation has been used in cell work, and to lesser extent transgenic lines. However, in clinical settings, this mutation has been associated with a later age of onset (54.2-64.2 years of age) and exhibits less toxicity that allows for a longer duration of disease (up to 20 years) (Van Swieten et al., 1999). Reed et al. (1997) conducted a brain autopsy from an individual with a long family histology of the R406W mutation. Out of four generations, there were 27 individuals and approximately 56% were affected by the mutation. Electron microscopy evidenced paired helical filaments (PHFs) in the midbrain regions, neurofibrillary tangles (NFTs), and ghost tangles in the hippocampal region, and cortical atrophy. The family’s clinical symptomology included memory loss, personality change, and mild parkinsonism.

Because there are several mutations of tau that are related to tauopathies, it is important to compare how these mutations model tauopathy. Therefore, the purpose of this study was to use AAV9 methodology to compare 2N4R wild-type tau, tau$^{\text{P301L}}$, and tau$^{\text{R406W}}$ to examine which model may give the most translational applications for AD models in three aims.
Aim One: To investigate whether the amount of tauopathy is affected by tau variants.

Aim one hypotheses: Tau^{P301L} is shown to have an earlier onset and more severe progression when compared to tau^{R406W} in clinical cases. Furthermore, in animal work tau^{P301L} has more disease pathology when compared to tau^{wild-type}. Because of the increased pathology severity, it is hypothesized that the tau^{P301L} will produce more tauopathy compared to the other forms of tau.

Aim Two: To compare other markers of tau-related pathology such as immune activation and neurodegeneration to determine which virus produces a robust model of tauopathy.

Aim two hypotheses: Given tau^{P301L} is more prone to aggregation, it would be expected that this would be coupled with an increased immune response and have more of indicators of neurodegeneration, such as a reduction in hippocampal volume, and neuronal density.

Aim Three: To evaluate the if the tau variants can induce cognitive impairments

Aim three hypothesis: Because tau^{P301L} is more prone to aggregation, in addition to more tau burden and tau-related pathology, it is anticipated that the tau^{P301L} will perform worse on measures of memory.

Method

Animals and Experimental Design

Female (n=20) and male (n=20) C57BL/6 mice were ordered from the National Institute of Aging. Animals were kept on a 12-hour light cycle and were provided food water ad libitum. All animals were aged to 12 months and received intracranial stereotaxic surgeries to bilateral hippocampi (coordinates ML: +/-2.7, AP:-2.7, DV:-3.0) and anterior region of the cortex.
(coordinates ML:+/-2.20, AP:2.2, DV:-3.0) relative to bregma using a glass pulled pipette needle attached with heat shrink tubing to a 10μL Hamilton syringe (Hamilton, Pompton Plains, NJ; cat. no. 80000) at a rate of 0.5μL/minute. Following the surgery, animals were placed on heating pads and observed during recovery. Weights were measured weekly to confirm health status of mice during the duration following the surgeries until tissue collection. At 3.5 months of viral incubation, behavioral assessments were conducted to observe any deficits in memory. Brain tissue was collected two weeks following behavioral assessments. (Figure 19)

**Viral Constructs**

Glycerol stocks of competent SURE2 cells containing 2N4R tau\textsuperscript{wild-type} and GFP plasmids were donated by Dr. Kevin Nash from the University of South Florida. The gene fragments for both 2N4R tau\textsuperscript{P301L} and 2N4R tau\textsuperscript{R406W} were ordered from GeneWiz (Genewiz, South Plainfield, NJ, USA). However, only the GFP plasmid contained the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) an important element that can enhance expression of viral cargo genes and elongate the period of viral expression (Loeb et al., 1999). To ensure the plasmids were consistent and included WPRE, three additional GFP plasmids were prepared and restriction digests were conducted at the AgeI and SalI sites to remove the GFP fragments. Ligations were conducted to insert each of the tau plasmids (P301L, R406W, and wild-type). To ensure expression in the CNS each expression plasmid contained cytomegalovirus (CMV) enhancer with the chicken β actin (CBA) promoter (Gray et al., 2011). The plasmids were transfected with AAV9 plasmid and XX6 helper plasmid in HEK293T cells. Viruses were purified using iodixanol gradients and ultracentrifugation. Further steps were conducted to concentrate the virus with Amicon ultra-15 centrifugal filters (Sigma, Burlington, MA, USA; Cat. No. UFC910024) with DPBS (pH 7.4). Ten μL aliquots were made and stored in the -80°C.
until the day of surgery to eliminate freeze thaw cycles. RT qPCR were used for viral titering with primers for the WPRE sequence. Each virus was diluted to $8.8 \times 10^{11}$ vector genomes (vg)/uL. Furthermore, $8.8 \times 10^{10}$ vg/mL of AAV9 GFP was added to each of the tau viruses so the area of injection could be monitored with GFP staining.

**Behavioral Assessments**

All behavioral tests were conducted by an experimenter blind to tau variant injection group of the mice. Open field assessment was conducted to determine general activity and to acclimate the animals to behavioral handling. Animals were placed in the open field (L 40mm/W 40mm/H 40mm) enclosure from BioSeb (Vitrolles France) and tracked with AnyMaze software (Stoelting, Wood Dale, IL, USA; cat. no. 60000) for 10-minute trials. The total distance traveled was measured. Rotarod was used to evaluate the animal’s motor skill and to identify any potential motor impairments. This test was conducted over two days with 4 trials on each day. Animals were placed on the TSE (TSE Systems, Chesterfield, MO, USA) rotarod apparatus, which is a rotating rod that increases in speed through the duration of the 5-minute trial. There was at least 20 minutes between each trial to minimize muscle fatigue over trials. The latency to fall was recorded for analysis.

To evaluate visuospatial navigation memory the radial arm water maze (RAWM) was conducted as described previously (Alamed et al., 2006). This test was conducted over four days and utilized a 6-arm maze that radiates from the center that was filled with water and external cues that were placed on the wall. For each animal, the goal arm of the 6-arm maze that houses the platform remained the same, but the start arm changed with each trial. Furthermore, the goal arm was changed between animals to avoid any overt olfactory cues. Across all four days, each animal underwent 15 trials for 1 minute each. On day one, mice were trained by alternating
between a visible and hidden platform. Furthermore, if the mouse failed to reach the platform on day one after the 1-minute trial, the animal was guided to the platform. The number of incorrect arm entries that included all four limbs to be in the arm were recorded as errors. Day two involves only the hidden platform, again, incorrect arm entries were noted. Day three measured reversal learning. The hidden platform was moved to the arm 180° across the maze from the original goal arm. Finally; on day four, mice were tested for capability to see, swim towards, and ascend the visual platform. The visible cues were removed from the wall and the visible platform was placed in the center of the maze. No flag was used. The latency to reach the visual platform was recorded. The number of errors and platform latencies were averaged in sets of three to create 5 blocks of trials each day.

Tissue Collection

Four months after the injection of the AAV, tissues were collected in an identical manner as the previous studies. The mice were anesthetized with 0.1mL/10g Somnasol (0.25mL in 10mL of H2O) and placed on a heating pad to maintain body heat to reduce any artefactual tau phosphorylation caused by reduced body temperature during anesthesia (Planel et al., 2007). Animals were perfused transcardially with 25mL of 0.9% normal saline solution. Once the animals were perfused, the brains were immediately extracted. The brains were placed on an iced stage and the right hemisphere was dissected into the anterior region of the cortex (ACX), posterior region of the cortex (PCX), and the hippocampus (HPC) and brain regions were immediately frozen on dry ice. Brain region specimens were stored at -80°C until homogenization for biochemical analysis. The left hemisphere was immediately immersed in 20mL of freshly prepared 4% paraformaldehyde (pH 7.4) for 24 hours at 4°C. After 24 hours, the left hemispheres were cryoprotected, where each brain was placed successively in 10, 20, and
30% sucrose for 24 hours. The brain hemispheres were stored at 4°C until sectioned. Brains were horizontally sectioned with a freezing microtome at a 25μm thickness and placed into Dulbecco’s phosphate buffered saline with 10 mM sodium azide solution (7.4 pH) except for every 10th section that was sectioned at 50μm thickness for hippocampal volumetric analysis.

**Tissue Homogenization and Biochemical Analysis**

The HPC was added 10v/w to RIPA buffer (50mM Tris-Cl, 150nM NaCl, 1% NP40, 0.1% SDS, 0.05% sodium deoxycholate) buffer containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P8340), deacetylase inhibitor cocktail (MedChem Express, Monmouth Junction, NJ, USA; Cat. No. HY-K0030) phenylmethanesulfonyl fluoride (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P7626), and phosphate inhibitor cocktails II and III (Sigma Aldrich, St. Louis, MO, USA; Cat. Nos. P5726 & P0044, respectively). Homogenization was conducted by first using a mechanical pestle and then by sonication. An aliquot of the crude homogenate was centrifuged at 50,000g at 4°C for 1 hour. The supernatant was removed and stored at -80°C as the detergent soluble fraction. The detergent insoluble pellet was treated with 70% formic acid. Once treated with formic acid, the pellet was then homogenized with the mechanical pestle. After the pellets were homogenized, the samples were gently rotated for 30 minutes. 1M Tris-Base (pH 7.6) was added to each sample and then neutralized with 15.1M NaOH. Individual samples were adjusted to pH of 7. Pierce BCA protein assays were done to determine the protein concentration of both the detergent soluble and insoluble fractions.

Samples for both the detergent soluble and insoluble fractions were used for all tau ELISA assays. Assays were used to detect total tau (Fisher Scientific, Cat. No. KHB0041), phosphorylated Ser199-tau (Fisher Scientific, Cat. No. KHB7041), and phosphorylated Ser396-
tau (Fisher Scientific, Cat. No. KHB7031). All samples were first diluted (1:100) in RIPA, then
diluted appropriately for each ELISA assay in the provided ELISA kit’s buffer. All
concentrations derived from the ELISA assay were normalized to the sample’s total protein
concentration.

**Histopathology**

Six to eight sections from each mouse were placed in free-floating multi-sample staining
trays for each marker. The trays were placed in an endogenous peroxidase blocking solution
(10% methanol, 3%H\textsubscript{2}O\textsubscript{2} in PBS). The tissues were washed 3 times with PBS and then placed in
permeabilizing solution (0.2% lysine, 1%Trition X100, 4% normal goat serum in PBS). Tissues
were then incubated overnight in the appropriate antibody (see Table 3). The following morning,
tissues were washed with PBS and incubated for 2 hours in the corresponding biotinylated
secondary antibody. This step was excluded if the primary antibody was biotinylated. After the
two-hour incubation period, tissues were washed with PBS and placed in Vectastain® Elite®
ABC kit solution (Vector Laboratories, Burlingame, CA; cat. no. PK-6100) to enhance the
enzyme complex. Again, tissues were washed with PBS for 2 washes and the final wash was in
TBS. Following the TBS wash, tissues were placed in a color development solution (0.05%
DAB, 0.5% Ni\textsuperscript{2+}, .03% H2O2) for 5 minutes. To stop color development, tissues were washed
with TBS for one wash and then 2 PBS washes. Upon the completion of staining, tissues were
mounted on microscope slides (Fisher Scientific, Waltham, MA; cat. no. 1255015), dehydrated,
and cover slipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA;
cat. no. 13512).

For Gallyas and cresyl violet staining, tissue sections were first mounted on microscope
slides and dried overnight. Gallyas staining is a silver stain that identifies NFTs (Uchihara,
On the day of Gallyas staining, sections were briefly rehydrated and then placed in a pretreatment solution (5% periodic acid). After a series of washes, the slides were immersed in a silver enhancing solution (10% potassium iodide, 4% NaOH, 0.035% silver nitrate) for one minute before incubating for 10 minutes in a 0.5% acetic acid solution. Slides were then placed in a working developer solution (2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.5% tungstosilic acid, 0.2% formaldehyde) for approximately 10 minutes. Upon development, slides were washed with 0.5% acetic acid followed by water washes. Then slides were immersed in a gold toning solution (0.1% gold chloride solution) followed by an additional water wash. Then slides were incubated in a solution to remove traces of unconjugated silver salts (0.1% sodium thiosulphate solution) and a final water wash. Slides were immediately dehydrated and coverslipped.

The cresyl violet stain was conducted on pre-mounted 50μm tissues. The day of the stain, slides were briefly rehydrated and then placed in a 0.05% cresyl violet dye solution. After staining, slides were briefly placed in an acetic acid water solution (pH 3.5) and rinsed in water. Slides were then dehydrated and coverslipped in DPX (Electronic Microscopy Sciences Cat. No. 13512). Once the DPX was dried, slides were scanned using ZEISS Axio Scan.Z1. Scanned slides were analyzed using NearCyte tissue analysis IAE software (created by Andrew Lesniak). The entire tissue sections were identified and immunopositive pixels above a user-defined threshold were measured. The threshold parameters were derived using several sections with light and dark staining and held constant throughout the remaining analysis. The fraction of the total region occupied by immunopositive pixels was calculated. Cresyl violet staining was conducted to measure hippocampal volume using the method of Cavalieri.
**Statistical Analysis**

All statistical analyses were conducted using IBM SPSS Statistics 25 (Armonk, New York, USA) and figures were created with GraphPad Prism 8 (La Jolla, CA). For aim one, one-way ANOVAs were conducted to determine the group differences based on injection group (GFP, tau$^{P301L}$, tau$^{R40w}$, and tau$^{\text{wild-type}}$) for markers of tauopathy from histological staining and ELISA assays concentrations. In addition, one-way ANOVAs were also used to identify any group differences based on injection group (GFP, tau$^{P301L}$, tau$^{R406w}$, and tau$^{\text{wild-type}}$) for tau-related pathology including histological markers of inflammation and neurodegeneration were conducted for aim two. Furthermore, to assess any group differences on measures of the behavioral assessments for aim three, both a one-way ANOVA, and one-way repeated measures ANOVAs were conducted. First the open field was analyzed by one-way ANOVA for any group differences based on injection group (GFP, tau$^{P301L}$, tau$^{R40w}$, and tau$^{\text{wild-type}}$) for the total distance traveled during a 10-minute trial. Because the rotarod task is conducted in eight trials over two consecutive days, a repeated measures one-way ANOVA was conducted to determine any group differences based on injection group (GFP, tau$^{P301L}$, tau$^{R40w}$, and tau$^{\text{wild-type}}$) for the time to the animal was able to remain on a rotating rod over the two days of testing. Finally, the RAWM has five blocks of trials on each day of the tasks and to identify any groups differences based on injection group (GFP, tau$^{P301L}$, tau$^{R40w}$, and tau$^{\text{wild-type}}$) for task performance by the average of error made, one-way repeated measures ANOVAs were conducted for day one, day two, and the reversal task. For the open pool task, time to reach the platform was analyzed by a one-way repeated measures ANOVA to determine any group differences based on injection group (GFP, tau$^{P301L}$, tau$^{R40w}$, and tau$^{\text{wild-type}}$) to reach the platform through all five blocks of trials. All
significant ANOVAs were followed by Fisher’s least significance difference (LSD) post-hoc analyses to identify the significantly different groups.

Results

Aim One: Effect of tau variants on tauopathy

GFP and total tau expression

The goal of this study was to compare three different tau viruses to determine which virus will produce a more translational model of AD tauopathy. To define the brain area affected by the intracranial injections, each tau virus included a 1/10th titer of GFP relative to the GFP injected mice. Representative images for both GFP and total tau staining can be seen in Figure 20. Histological GFP was examined by one-way ANOVA to determine if there were significant differences between GFP, tau$^{\text{P301L}}$, tau$^{\text{R406W}}$, and tau$^{\text{wild-type}}$ injected animals. There were significant differences found, $F(3,35) = 130.538$, $p < .001$ (Figure 21A) and Fisher’s LSD post-hoc analysis determined that GFP injected mice had significantly greater GFP staining compared to tau$^{\text{P301L}}$ ($p < .001$), tau$^{\text{R406W}}$ ($p < .001$), and tau$^{\text{wild-type}}$ ($p < .001$). In addition, tau$^{\text{R406W}}$ had significantly more GFP staining compared to tau$^{\text{P301L}}$ ($p < .001$) and tau$^{\text{wild-type}}$ ($p < .001$). To measure histological total tau, HT7 was used for staining. A one-way ANOVA found significant differences between GFP and the tau viruses ($F(3,35) = 73.580$, $p < .001$; tau$^{\text{P301L}}$ ($p < .001$), tau$^{\text{R406W}}$ ($p < .001$), and tau$^{\text{wild-type}}$ ($p < .001$), Figure 21B). Out of the tau virus groups, tau$^{\text{wild-type}}$ had the greatest amount of HT7 staining and was significantly more when compared to tau$^{\text{P301L}}$ ($p < .001$) and tau$^{\text{R406W}}$ ($p < .001$) (Figure 20E-H). Total tau concentrations were detected by ELISA assays for both the detergent soluble and detergent insoluble fractions. For soluble fraction total tau, a one-way ANOVA detected significant group differences ($F(3,35) = 27.556$, $p$
< .001; Figure 21C). Fisher’s LSD post-hoc analysis found significant differences between GFP and two of the tau viruses, tau^{P301L} (p < .001) and tau^{wild-type} (p < .001). Additional differences were detected between tau^{P301L} and tau^{R406W} (p < .001) where there was significantly less total soluble tau in the tau^{R406W} (p < .001) injected animals. In fact, there was no significant difference between tau^{R406W} and GFP (p = .150). Because of the low tau^{R406W} concentration, there was also a significant difference between tau^{R406W} and tau^{wild-type} (p < .001). The detergent insoluble fraction total tau concentration had similar group differences as the soluble fraction (F(3,33) = 19.531, p < .001; Figure 21D). There were significant differences between GFP and two of the tau viruses, tau^{P301L} (p < .001) and tau^{wild-type} (p = .019). Furthermore, the tau^{P301L} had a significantly greater concentration when compared to the other tau viruses tau^{R406W} (p < .001) and tau^{wild-type} (p < .001). There were two animals that did not have enough insoluble sample to run in the ELISA assays that included one tau^{P301L} injected, and one tau^{wild-type} injected mouse. These two animals were not included for any of the detergent insoluble analyses.

Phospho-tau epitopes

To further examine markers of tauopathy, markers of early and late phosphorylation epitopes were examined (Figure 22). Histological AT8 was used as an early marker of phosphorylation and significant group differences were observed (F(3,35) = 29.652, p < .001). Fisher’s LSD post-hoc analysis revealed significant differences between tau^{wild-type} and all other viruses (ps < .001), as tau^{wild-type} had the greatest amount of AT8 staining. In addition, there were significant differences between GFP and tau^{R406W} (p = .007), but the difference between GFP and tau^{P301L} failed to reach significance (p = .201). When examining phospho-tau Ser199 (pSer199) concentration in the detergent soluble fraction, a one-way ANOVA revealed significant differences (F(3,35) = 27.556, p < .001) between tau variants. It was evident that there were
significant concentrations of tau$^{\text{P301L}}$ and tau$^{\text{wild-type}}$. Tau$^{\text{wild-type}}$ had the greatest detergent soluble pSer199 concentration and was significantly different from tau$^{\text{P301L}}$ ($p = .010$), tau$^{\text{R406W}}$ ($p < .001$), and GFP ($p < .001$) injection groups. Furthermore, tau$^{\text{P301L}}$ was significantly different from tau$^{\text{R406W}}$ ($p = .001$) and GFP ($p < .001$). While there was a low concentration of tau$^{\text{R406W}}$ detected, it was not significantly different from the GFP ($p = .184$) injected animals. Similar to the soluble fraction, pSer199 concentration in the insoluble fraction was significantly elevated in tau$^{\text{P301L}}$ and tau$^{\text{wild-type}}$ injected mice. However, for the insoluble fraction, tau$^{\text{P301L}}$ had the highest pSer199 concentration and was significantly different from GFP ($p < .001$), tau$^{\text{R406W}}$ ($p < .001$), and tau$^{\text{wild-type}}$ ($p = .032$) groups. Additionally, tau$^{\text{wild-type}}$ had significantly greater concentrations of insoluble pSer199 when compared to GFP ($p = .014$) and tau$^{\text{R406W}}$ ($p = .027$). Consistent with the soluble fraction, tau$^{\text{R406W}}$ was not significantly different from the GFP ($p = .818$) injected groups.

Phospho-tau Ser396 (pSer396) was examined as an indicator of late phosphorylation. Histological pSer396 had a significant main effect of tau variant, $F(3,35) = 8.753, p < .001$ (Figure 20M-P). Tau$^{\text{wild-type}}$ had the greatest amount of staining and was significantly different from GFP ($p < .001$), tau$^{\text{P301L}}$ ($p = .033$), and tau$^{\text{R406W}}$ ($p < .001$) injection groups. Furthermore, tau$^{\text{P301L}}$ had significantly more pSer396 staining compared to GFP ($p = .017$). There was a low amount of pSer396 staining in the tau$^{\text{R406W}}$ injected animals, but this was not significantly different from the GFP ($p = .501$) injected animals. The detergent soluble pSer396 concentration was comparable to histological pSer396 ($F(3,35) = 22.166, p < .001$), where tau$^{\text{wild-type}}$ had the greatest concentration and was significantly greater than all other viruses ($ps < .001$). Furthermore, tau$^{\text{P301L}}$ had the second highest concentration and was significantly different from GFP ($p = .003$) and tau$^{\text{R406W}}$ ($p = .008$). The tau$^{\text{R406W}}$ failed to have a significant difference from
the GFP injected animals ($p = .804$). While the pSer396 concentration of the insoluble fraction had significant differences ($F(3,33) = 12.787, p < .001$), tau$^{P301L}$ had the highest concentration was the significantly greater than all other groups ($ps < .001$).

**Neurofibrillary tangles**

Gallyas staining was used to examine if there was a significant difference in silver positive NFTs based on the tau variant injection group. A one-way ANOVA was conducted and revealed significant differences among the groups ($F(3,35) = 23.132, p < .001$; Figure 23). Only tau$^{P301L}$ ($p < .001$) and tau$^{\text{wild-type}}$ ($p = .028$) had Gallyas staining that was significantly different from the GFP injected animals. Furthermore, tau$^{P301L}$ had the greatest amount of Gallyas staining when compared to the tau$^{\text{wild-type}}$ ($p < .001$) injected animals.

**Aim Two: Effects of tau variant on other markers of tau-related pathology**

**Microglia**

Iba1 staining was used to monitor microglia in each injection group. A one-way ANOVA was conducted (Figure 24A). There were no significant differences based on the injection group ($F(3, 35) = 2.067, p = .122$). However, when examining microglial activation using MHCII marker, there were significant group differences ($F(3,35) = 14.478, p < .001$; see Figure 24B). Tau$^{\text{wild-type}}$ had the greatest amount of MHCII staining when compared to all other injection groups ($ps < .003$). The only other significant difference was observed for tau$^{R406W}$ and GFP injection groups, where tau$^{R406W}$ had significantly greater MHCII staining when compared to the GFP ($p = .006$) injection group.
Neurodegeneration

When examining the histological neuronal marker, NeuN, there was a significant injection group difference ($F(3,35) = 6.840, p = .004$; Figure 25A). Fisher’s LSD found that GFP and tau$^{P301L}$ had significantly less neuronal staining when compared to tau$^{R406W}$ ($ps < .003$) and tau$^{\text{wild-type}}$ ($ps < .04$). Furthermore, there was a main effect of injection group on hippocampal volume, ($F(3,35) = 5.668, p = .003$), and tau$^{\text{wild-type}}$ had a significant reduction in hippocampal volume when compared to all other viruses ($p < .0095$; Figure 25B).

Aim Three: Effect of tau variant on activity, locomotor, and cognitive performance

Open field and rotarod

The first behavioral measure that was assessed was the open field as an indicator of general activity. The total distance traveled over a 10-minute trial was determined by a one-way ANOVA. There were no significant differences observed between the tau variants, $F(3,33) = .122, p = .946$ (Figure 26). Two tau$^{R406W}$ injected animals were excluded from the open field assessment because of a technical issue with the software. Similarly, rotarod performance had no significant difference between the tau variants ($F(3,35) = .928, p = .437$; Figure 27).

Radial arm water maze

To evaluate any evidence of cognitive impairment based on injection group differences, the RAWM task was performed and analyzed by repeated measures two-way ANOVAs for each day of testing. For day one, the training day, there was no evidence of differences based on the injection group, $F(3,23) = .796, p = .508$ (Figure 28A). However, on the testing day, or day two, there was a main effect of tau variant injection group ($F(3,23) = 7.716, p = .001$; Figure 28B). Fisher’s LSD post-hoc analysis determined that the tau$^{P301L}$ performed significantly more errors.
when compared to the GFP \((p = .025)\) and the \(\text{tau}^{\text{R406W}} (p = .003)\) injection groups. In addition, the \(\text{tau}^{\text{wild-type}}\) also performed significantly more errors when compared to GFP \((p = .004)\) and \(\text{tau}^{\text{R406W}} (p < .001)\) but was not significantly different from the \(\text{tau}^{\text{P301L}} (p = .571)\) group. When working memory, as demonstrated by the reversal task of the RAWM, was examined for any evidence of injection differences, there was no significant main effect of tau variant injection group on the amount of errors \((F(3,23) = 1.572, p = .223; \text{Figure 29A})\).

All animals were evaluated for indication of visual impairment by the open pool task. If any animal took longer than 20 seconds to find the visual platform on the last trial, the animal was believed to have a visual impairment and the animal was excluded from any RAWM analysis. For the animals that were able to complete the task in 20 seconds or less, a repeated measures ANOVA was conducted to determine if there were any differences based on injection group (Figure 29B). Fisher’s LSD post-hoc analysis indicated that the only group that performed significantly different, was the \(\text{tau}^{\text{wild-type}}\) injection group \((ps < .002)\), where this group required significantly more time to reach the visible platform than the other three groups.

There was one animal that died prior to the behavioral assessments. An additional 9 animals were excluded from RAWM analysis. Of the 9 animals, 2 had eye problems, 2 were not able to swim, and 5 were noted to have seizures on the days of testing. In addition, three animals were excluded due to not being able to reach the visible platform in 20 seconds on the last trial of the open pool task. This resulted in final sample sizes of GFP \(n = 7\), \(\text{tau}^{\text{P301L}} n = 5\), \(\text{tau}^{\text{R406W}} n = 9\), and \(\text{tau}^{\text{wild-type}} n = 6\) for the radial arm water maze.
Discussion

This study aimed to understand the difference in the capacity of three tau genetic variants that included tau^{P301L}, tau^{R406W}, and full length tau^{wild-type}, for their ability to produce a translational model of AD tauopathy. There were three aims that this study was designed to address. Aim one was to determine if there were differences in tauopathy based on the injection group. It was evident that tau^{P301L} and tau^{wild-type} viruses were able to produce models of tauopathy. While both injection groups were able to induce tauopathy, the two groups did so in different ways. The tau^{P301L} virus had the highest concentrations of early and late phosphorylated tau in the detergent insoluble fractions. Furthermore, the tau^{P301L} also had the most abundance of NFT staining measured by Gallyas staining. These findings resonate with the enhanced aggregation propensity for tau^{P301L} relative to tau^{wild-type}. In comparison, the tau^{wild-type} injection group had significantly greater total tau staining coupled with higher concentrations of early and late phosphorylated tau in the detergent soluble fractions.

While tau^{P301L} and tau^{wild-type} viruses demonstrated models of tauopathy, the tau^{R406W} injection group failed to demonstrate a sufficient model of tauopathy. The expression of soluble total tau and histological total tau demonstrated that the intracranial injections were successful; however, this group only had evidence of early phosphorylated tau in the histological probe, and overall expression appeared reduced. Research has demonstrated that this mutation has a less severe progression of pathology (Van Swieten et al., 1999). Intriguingly, this injection group also had the greatest amount of GFP staining among the tau viruses. However, with the greater amount of GFP staining, one possibility is there is less competition between the GFP and tau^{R406W} viruses for cellular resources than for the other tau variants.
Studies that have examined the in vitro differences of 2N4R mutant and wild-type tau in their ability to aggregate and polymerize (Mutreja, Combs, & Gamblin, 2018). Tau\textsuperscript{P301L} exhibited a greater degree of aggregation polymerization when compared to tau\textsuperscript{wild-type} and tau\textsuperscript{R406W} groups. In addition, tau\textsuperscript{P301L} proved to have the most reduction in microtubule assembly and stabilization when compared to tau\textsuperscript{R406W} and tau\textsuperscript{wild-type}. Electron micrographs demonstrated that while tau\textsuperscript{P301L} had a lower number of filaments, tau\textsuperscript{P301L} had the longest filaments in comparison to both tau\textsuperscript{R406W} and tau\textsuperscript{wild-type}. These findings specific to the P301L mutation are consistent with the increased amount of Gallyas staining and concentrations of insoluble tau found in the tau\textsuperscript{P301L} injection group observed in the current study.

Aim two was to examine injection group differences on markers of tauopathy-related pathology. When examining markers of immune function, there were no group differences on total microglia staining, but there was evidence of group differences for activated inflammation by MHCII staining. Tau\textsuperscript{wild-type} had the greatest amount of MHCII activation that was coupled with the greatest amount of hippocampal atrophy. All of the tau viral groups had a reduction in hippocampal volume, but it was the most striking in the tau\textsuperscript{wild-type} injection group, nearing a mean difference of 50% compared with GFP injected mice. When examining a neuronal marker, the results were unexpected. The GFP and tau\textsuperscript{P301L} injected groups had the least amount of NeuN staining while the tau\textsuperscript{wild-type} and then tau\textsuperscript{R406W} had the greatest amount of neuronal staining. As the tau\textsuperscript{R406W} injected group displayed the least amount of pathology of the tau injection groups, it is expected that this group would have a high level of neuronal staining. This suggests that changes in volume are larger than changes in neuron number. Changes in volume could reflect loss of fibers, synapses and/or glial components. If the neuron number is not reduced but the hippocampal volume is reduced, then those neurons would be present in a smaller volume.
leading to the observed effects. Thus, tau$^{\text{wild-type}}$ and tau$^{R406W}$ might express more synapse/fiber toxicity, while tau$^{P301L}$ and GFP are more toxic to neurons.

When examining the findings of aim three to identify potential behavioral impairments, it was apparent that there were no significant differences in motor function across the injected groups as demonstrated by the open field and the rotarod tests. However, group differences based on the injection groups were observed on the RAWM. On the testing day, both the tau$^{P301L}$ and tau$^{\text{wild-type}}$ injected mice performed significantly worse on the memory task when compared to the GFP and the tau$^{R406W}$ injected groups. When open pool was examined for the animal’s ability to reach the visible platform among animals without visual impairment, the tau$^{\text{wild-type}}$ injection group took longer to reach the platform. This increased latency to reach the platform may indicate that it took longer for these animals to orient themselves to the center of the maze. It is important to note that the two groups that experienced cognitive impairments, also had the greatest number of animals excluded from analysis.

The overall evidence of this study shows that both tau$^{P301L}$ and tau$^{\text{wild-type}}$ viruses were sufficiently able to induce models of tauopathy that are consistent with the literature (Cook et al., 2015; Jaworski et al., 2009). However as previously discussed, there are different mechanisms that are accumulating in that pathology, where both mechanisms can be translated to behavioral measures. This area warrants further examination. One hypothesis is that the tau$^{\text{wild-type}}$ injection could be inducing oligomeric tau whereas the tau$^{P301L}$ injection produces NFTs. Tau oligomers have been found to be a toxic form of tau that occurs early in the progression of pathology (Lasagna-Reeves et al., 2012; Maeda et al., 2006; Shafiei et al., 2017). Work by Maeda, Sato, and Takashima (2018) observed significant differences in the structure of in vitro oligomers that are formed in respect to tau$^{\text{wild-type}}$, tau$^{P301L}$, and tau$^{R406W}$. The most remarkable difference was
observed for the tau$^{P301L}$ where the oligomers were smaller in diameter. Furthermore, they found a significantly greater numbers of granules for the mutated forms of tau. This study provides evidence that there are inherent differences that should be expected for each of the tau viruses regarding oligomers.

In respect to lack of pathology in the tau$^{R406W}$ injection group, research has shown that this mutation causes pathology to progress at a slower rate specifically when compared to the tau$^{P301L}$ mutation (Van Swieten et al., 1999). This evidence demonstrates tau$^{R406W}$ as an ineffective model of tauopathy due to the lack of significant differences between the GFP injected group, and limited evidence for early phosphorylated tau.

Overall, the most surprising observation is that the wild type tau caused the greatest toxicity in terms of tissue atrophy. One criticisms of the existing transgenic mouse models of tauopathy is that they are not models of AD, but most appropriately models of frontotemporal lobe dementia, the disorder caused by these mutations. The results of the present study, using older mice than most such analyses, suggests that AAV9 tau$^{\text{wild type}}$ might be used to study tau pathology that is more closely related to that found in AD than in frontotemporal lobe dementia.

The evidence that comparing different viral tau isoforms in middle-age, demonstrates the importance of incorporating age in animal models of AD. Incorporating age has the potential to increase the modeling’s translational relevancy to clinical cases as it is an age-related disease. Furthermore, the comparison of various models of tauopathy to determine a more relevant AD model is important as the field has not identified an AD specific model tauopathy as observed in clinical cases. As there is no method of the intervention for the disease, it is important to identify more translationally meaningful models to identify potential areas for future interventions or preventative approaches that may be more advantageous.
In conclusion this study provided evidence that both tau\textsuperscript{P301L} and tau\textsuperscript{wild-type} viruses created two different models of tauopathy that included similar translational behavioral deficits. Tau\textsuperscript{P301L} produced an abundant amount of tauopathy that is less degradable in the insoluble forms of tau and NFTs. Tau\textsuperscript{wild-type} produced more soluble forms of tauopathy but a striking amount of atrophy. These differences should be further investigated to determine the cause of these differences to further determine the AD tauopathy translation ability of these models.
Figure 19. Experimental design and flowchart with sample sizes for the comparison of intracranial viral GFP, tau^{p301L}, tau^{R406W}, and tau^{wild-type} injections. Red boxes indicate attrition due to mortality, health concerns, or inability to swim. GFP= green florescent protein (control virus).
Table 4.

Summary of Primary and Secondary Antibodies Used for Immunohistochemistry

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<th>Host</th>
<th>Target</th>
<th>Source</th>
<th>Dilution</th>
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<tr>
<td>HT7</td>
<td>Mouse</td>
<td>Total Tau</td>
<td>Invitrogen MN1000B</td>
<td>1:5k</td>
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<tr>
<td>AT8</td>
<td>Mouse</td>
<td>Phosphorylated tau at Serine 202 and threonine 205 epitopes</td>
<td>Invitrogen MN1020B</td>
<td>1:10k</td>
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<tr>
<td>pSer396</td>
<td>Rabbit</td>
<td>Phosphorylated tau at the Serine 396 epitope</td>
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<td>I-A/I-E</td>
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<td>Major histocompatibility complex II</td>
<td>BD Pharmingen 556999</td>
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<tr>
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<td>Rabbit</td>
<td>Microglia</td>
<td>Fuji 019-19741</td>
<td>1:5k</td>
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<tr>
<td>NeuN</td>
<td>Mouse</td>
<td>Neurons</td>
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<table>
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<th>Manufacturer</th>
<th>Dilution</th>
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<td>Biotinylated Anti-Rabbit IgG</td>
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<td>Conjugates to Rabbit host primary antibodies</td>
<td>Vector Laboratories BA-1000</td>
<td>1:3k</td>
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<tr>
<td>Biotinylated Anti-Rat IgG</td>
<td>Goat</td>
<td>Conjugates to Rat host primary antibodies</td>
<td>Vector Laboratories BA-9400</td>
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<tr>
<td>Biotinylated Anti-Chicken IgY</td>
<td>Goat</td>
<td>Conjugates to Chicken host primary antibodies</td>
<td>Vector Laboratories BA-9010</td>
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Figure 20. Micrograph representation of the hippocampal area for histological GFP and tau staining. A-D, GFP staining; E-H, total tau measured by HT7; I-L phosphorylated tau at ser202/thr205 (AT8); M-P, phosphorylated tau at ser396 (pSer396); Q-T, Gallyas staining. GFP injected mouse A,E, I, M,Q; B,F,J,N,R tau P301L injected mouse; C,G,K,O,S tau R406W mouse; D,H,L,P,T tau wild-type. Scale bar 500μm.
Figure 21. Scatterplots of individual animal’s expression of GFP and tau after intracranial injections of AAV9 GFP, AAV9 tau^{P301L}, AAV9 tau^{R406W}, and AAV9 tau^{wild-type}. (A) Quantification of histological positive fractional area for GFP staining; \( n = 39 \). (B) Quantification of positive fractional area for histological total tau (HT7); \( n = 39 \). (C) The hippocampus was dissected, homogenized, and the detergent soluble fraction was isolated, and total tau ELISA assays to quantify total tau (HT7) concentration normalized to the total protein values; \( n = 39 \). (D) The hippocampal detergent insoluble fraction was isolated and used total tau ELISA assays to quantify total tau (HT7) concentration normalized to the total protein values; \( n = 37 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \( p < .001 \) * \( p < .05 \) Fisher’s LSD post-hoc analysis.
Figure 22. AAV9 tau^P301L_ and tau^wild-type_ exhibit early and late phosphorylated indicators of tauopathy. (A). Quantification of the positive fractional area of histological phospho-tau pSer202/pThr205 (AT8) demonstrating an age-dependent increase in the tau^P301L_ injected animals; \( n = 39 \). (B) The pSer199 concentration measured by ELISA assays on the detergent soluble hippocampal fraction normalized to total protein; \( n = 39 \). (C) The pSer199 concentration measured by ELISA assays on the detergent insoluble hippocampal fraction normalized to total protein; \( n = 37 \). (D) Quantification of the positive fractional area of histological phospho-tau pSer396; \( n = 39 \). (E) The pSer396 concentration measured by ELISA assays on the detergent soluble hippocampal fraction normalized to total protein; \( n = 39 \). (F) The pSer396 concentration measured by ELISA assays on the detergent insoluble hippocampal fraction normalized to total protein; \( n = 37 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \( p < .001 \) ** \( p < .01 \) * \( p < .05 \) Fisher’s LSD.
Figure 23. AAV9 tau\textsuperscript{P301L} and AAV9 tau\textsuperscript{wild-type} produce Gallyas silver positive neurofibrillary tau tangles. Quantification of the positive fractional area for Gallyas staining by injection and age groups; \(n = 39\). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \(p < .001\) and * \(p < .05\) Fisher’s LSD post-hoc analysis.
Figure 24. AAV9 tau^{wild-type} had the greatest immune response measured by MHCII. Quantification of histological immune markers by age and injection. (A) Quantification of positive area ratio of pan histological microglia by Iba1 staining; \( n = 39 \). (B) Quantification of positive area ratio of histological MHCII expression microglia by MHCII staining; \( n = 39 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \( p < .001 \) * \( p < .05 \) Fisher’s LSD post-hoc analysis.
Figure 25. AAV9 Tau<sub>R406W</sub> and Tau<sub>wild-type</sub> have greater neuronal density but AAV 9 tau<sub>wild-type</sub> had significant atrophy at the injection sites. (A) Quantification of the positive fractional area for NeuN staining for injection groups; \( n = 39 \). (B) Quantification of hippocampal volume estimated using 8 sections through the entire region by the method of Cavalieri indicated that the tau<sub>wild-type</sub> mice had a significant reduction of hippocampal volume compared to the other injections; \( n = 39 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. *** \( p < .001 \), ** \( p < .01 \), * \( p < .05 \) Fisher’s LSD post-hoc analysis.
Figure 26. There was no difference in the distance traveled for the open field based on AAV9 injection type; $n=37$. Data are presented as mean ±SEM (error bars). Each dot represents one mouse.
Figure 27. No difference in the time spent on the rotarod by AAV9 injection group over the two days of testing; \( n = 39 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse.
Figure 28. AAV9 tau\textsuperscript{P301L} and AAV9 tau\textsuperscript{wild-type} performed worse on the second day of the radial arm water maze when compared to AAV9 tau\textsuperscript{R406W} and AAV9 GFP. (A) The five blocks of trials for day one of the radial arm water maze demonstrate no significant differences; \( n = 27 \). (B) The five blocks of trials for day one of the radial arm water maze demonstrated AAV9 tau\textsuperscript{P301L} and AAV9 tau\textsuperscript{wild-type} injected mice performing more errors than AAV9 tau\textsuperscript{R406W} and AAV9 GFP injected mice; \( n = 27 \). Data are presented as mean ±SEM (error bars). Each dot represents one mouse. * \( p < .05 \) Fisher’s LSD. RAWM = radial arm water maze.
Figure 29. There were no significant group differences on the radial arm water maze reversal task, but AAV9 tau wild-type took a significant longer time to reach the platform in the open pool task. (A). The number of errors made on the reversal task of the radial arm water maze for all injection groups, \( n = 27 \). (B) Time to reach the platform in the open pool task to ensure animals are free of visual impairments for all injection groups, \( n = 27 \). Results from one-way ANOVAs were conducted for each task. Data are presented as mean ±SEM (error bars). Each dot represents one mouse. ** \( p \leq .001 \) Fisher’s LSD post-hoc analysis. RAWM = radial arm water maze
CHAPTER FIVE:

DISCUSSION

This body of work has demonstrated the importance of evaluating age and genetic mutant variants of tau in translational models of tauopathy. As researchers are still trying to gain a full understanding of Alzheimer’s disease (AD) pathology to identify critical periods for interventions, animal models are crucial to help achieve these goals. Therefore, the evaluation of the current animal models is imperative to ensure their translational ability to clinical cases.

The present work showed evidence of age effects in both a transgenic and three viral models of tauopathy. For study one, markers of tauopathy, inflammation, and neurodegeneration differ through the life course. These findings are an extension of the early seminal age-series examinations of the model conducted by others (Ramsden et al., 2005; Santacruz et al., 2005). Dickey et al. (2009) demonstrated that not all markers in the rTg4510 line change in linear trends in an age-series examination up to ten months of age. This study lends support for non-linear modeling for indicators of pathology at ages older than those examined in Dickey et al. (2009). It is important to understand how these markers of tauopathy and tauopathy-related pathology differ across age to advanced ages, to adequately provide a robust examination of the model.

In addition to observing accumulation of pathology with age in the transgenic model, we also demonstrated that the age when tau pathology was initiated also influenced the extent of tauopathy. The viral modeling of tau$^{\text{P301L}}$ across young, middle-aged, and old mice shows a clear effect of the age of onset. All the tau$^{\text{P301L}}$ injected mice had evidence of tauopathy, but the old
animals that had a significantly greater amount of neurofibrillary tau tangles (NFTs). Clinical evidence has demonstrated that pathology can begin decades before the onset of clinical symptoms that usual manifest in older adulthood (Villemagne et al., 2008). This highlights the clinical relevance of inducing pathology at the ages of increased risk for clinical disease compared to transgenic models, which express an abundance of pathology at very early ages in the life course.

It is important to not only to examine the effect of the age at tauopathy onset, but it is also impairative to evaulate the various forms of tau to induce tauopathy. The present work compared the viral models using tau\textsuperscript{P301L}, tau\textsuperscript{R406W}, and tau\textsuperscript{wild-type} in middle aged (12 month) mice. These comparisons evidenced that the tau\textsuperscript{R406W} failed to demonstrate a substantial model of tauopathy. This mutation is known to have a slower pathological changes in comparison to other mutations (Van Swieten et al., 1999). This is consistent with the findings decribed here. The pathology observed in the viral model of tau\textsuperscript{P301L} was comparable to that in the transgenic rTg4510 model that shows pathological increases in detergent insoluble fractions and NFTs that are readily apparent. NFTs are a hallmark pathology of AD, so their presence in this model of tauopathy highlights its relevance. On the other hand, tau\textsuperscript{wild-type} injection produced changes in soluble tau that are accompanied by substantial reductions in hippocampal volume. Because tau\textsuperscript{wild-type} is associated with atrophy, it demonstrates a clear pathological event is taking place.

One important relevant cause may be tau oligomers. Early events in the progression of tauopathy should be further examined to understand the critical periods where these changes are occurring (Lasagna-Reeves et al., 2012; Maeda et al., 2006; Shafiei et al., 2017).
Limitations

No body of work is without limitations. This current dissertation has several limitations that included, first and foremost, the lack of the evaluation of tau oligomers. Furthermore, this body of work present evidence of a linear increase of NFTs, but other tau variants vary or even decrease with age. A current assumption is that ghost tangles can remain in tissue after neuronal death as a tombstone and could represent a toxic tau form. Evidence for ghost tangles was not evaluated. Therefore, currently we are not able to evaluate whether ghost tangles may be toxic. There are many tau variants that remain to be assessed in this model, including conformation-specific antibodies, tau phosphorylated at epitopes linked to Alzheimer’s disease, such as phospho-tau181, and tau with other post-translational modifications, such as truncations or acetylation. Assessment of tau oligomers and other tau variants in future experiments could contribute to understanding how tau variants affect neurodegeneration.

Limitations that are specific to study one includes the lack of behavioral measures. Behaviorial assessments were not performed because the rTg4510 line has been well characterized and demonstrated to exhibit cognitive impairments on a variety of tasks that include the radial arm water maze and novel object recognition among others (Brownlow et al., 2014; Joly-Amado et al., 2016; Ramsden et al., 2005; Santacruz et al., 2005). While cognitive impairments have been documented, the current work did not extend the findings to include behavioral measures, in part because these develop very early (4-6 months) in the course of tau accumulation. Work from Gamache et al. (2019) questioned the utility of the rTg4510 model, by demonstrating that the insertion of the P301L mutation can disrupt several genes that could impact the phenotype. However, the scope of the study was to understand a long-term age series examination of pathological indicators across the life course. This study was not able to examine
any impact caused by the potential gene disruption caused by the P301L transgene insertion. Yet, it is important to note that the changes in insoluble tau and NFTs were similar to the viral model of tauP301L.

Limitations for studies two and three are the lack of viral timecourse examination after tau viral injections. Given the severe hippocampal atrophy in the tauwild-type group, a time point shorter than four months might allow discrimination of more subtle group differences. On the other hand, a longer post-injection time points could allow effects of tau R406W to manifest. In addition, more detailed examination of important activated microglia changes could be pursued. The significant differences observed for age and tau variant injection for MHCII does provide support for differences in microglia activation.

Specifically in regards to study two, there was a large, unexpected amount of animals that were not able to swim through the four days of testing in the water maze. This caused a reduction in sample size and may have hindered detection of additional important differences. Furthermore, with aging animals, it is expected for some animals to have health concerns, but there were more animals than anticipated to have a health concern in this series of C57BL/6Nia mice (i.e., eye problems, seizures).

Future directions

To take the current findings and try to bridge the gap of the current limitations, future directions include the examination of any evidence of tau oligomers in the through the examination of ELISA or dot blot assays (Kanaan et al., 2016) in tau viral studies. Tau oligomers are hypothesized to induce neurotoxicity. Because argyrophilic tau aggregates are not
responsible for neurotoxicity in the tau<sup>wild-type</sup> injected mice, it is possible that more tau oligomers are produced in this condition.

Furthermore, to examine if any of the silver positive NFTs are evidence of ghost tangles, histological staining should be conducted on rTg4510 tissues to identify any ghost tangles. To identify the evidence of any ghost tangles would provide a complete evaluation of the model. In addition, to having a more detailed understanding of the rTg4510 model, evidence of ghost tangles may provide an explanation why some markers of tauopathy decrease with age.

To provide more detailed examinations of viral models of tauopathy, additional markers of reactive microglia should be examined. This may provide additional validation of this model. Finally, RAWM behavioral assessments with older mice should be evaluated to aid in the determination of what age that mice start to experience disruptions in their ability to swim. Alternative performance strategies could be considered such as fewer trials, longer inter-trial intervals or warmer water temperature to improve retention of aged mice. This is needed to reduce the likelihood of animals being excluded from future studies, or to establish increased numbers required to retain full statistical power.

The development of a viral delivery based model, such as described here, will provide researchers with an alternative to transgenic models. Transgenic models suffer from inability to initiate pathology at the age of risk. The strong AAV-based model described here recapitulates qualities of human disease. Its use will allow future researchers to explore mechanisms of neurodegeneration and to explore the translational potential of experimental therapeutics for Alzheimer’s disease and other tauopathies.
Implications for Health and Well-Being for Older Adults

To date there are no safe and effective treatments to reverse or halt the pathology that contributes to AD (Cummings et al., 2019; Cummings et al., 2014). However, previous research has clearly demonstrated that tau is more closely related to cognitive declines and neuronal loss associated with the disease (Bejanin et al., 2017; Gordon et al., 2018). Because of this relationship, this body of work was aimed at examining various models of tauopathy and their abilities to recapitulate features of human disease. Age remains the greatest risk factor for tauopathy and needs to be represented in a model for identifying markers for early detection and the treatments of AD. The present work examined age in two models of tauopathy. The transgenic model demonstrates an important shift in tau from the soluble, or less harmful forms of tau, to insoluble aggregates that are more progressed formations of pathology that can contribute to neuronal loss. This shift occurs around one year of age. Identifying this critical age period is important as this allows research to investigate the key changes for this pathological shift at the appropriate age. Identifying these key changes are critical as they can aid in the identification of early biomarkers and even new drug treatments for tau.

In addition, the viral modeling of tau allows for a model that is more relevant to human cases. For most AD diagnoses, pathology begins accumulating in mid to late life, not in early life and adulthood as demonstrated in transgenic animal models. Therefore, having a model that allows for the onset of the disease to occur in ages that are comparable to human cases provides important information to animal models. The aging immune system has a clear impact on the rate and formation of pathological tau, so to study this pathology, age of disease onset is a critical factor (Franceschi & Campisi, 2014; Joly-Amado et al., 2020; Lee et al., 2010; Licastro et al., 2005). Not only does implementing a later age of onset allow for a more meaningful display of
tau pathology but allows for the investigation of an aging immune system on the rate and formation of pathology. This is helpful as there are potential pathways in the aging immune system that may be more beneficial to target for drug treatments to reduce or halt pathological tau formations. Furthermore, having an aging model of pathology is more beneficial when administering drug treatments, as individuals that would receive treatments would be older in clinical settings and would be potentially impacted differently than younger individuals.

Because there are no treatments for AD, it is imperative for the animal studies to investigate the critical changes in tau and the immune system to identify key changes for the prevention and treatment of the disease. As demonstrated in this body of work, animal models are able to mimic features of clinical AD and provides the foundation for the additional work to aid in the identification of early changes. The examination of early changes have the ability to discover important early key biomarkers for the early detection of the disease as well as drug treatment targets that will allow for the discovery of a treatment that has the ability to aid the millions of individuals afflicted by AD.
REFERENCES


APPENDIX I:

IACUC APPROVALS

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM
TO: Marcia Gordon,

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 2/22/2017

PROJECT TITLE: Molecular Therapeutics to Mitigate Inflammation, Tauopathy and Degeneration

FUNDING SOURCE: Alzheimer's Association

IACUC PROTOCOL #: R IS00003384

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 2/22/2017:

Mouse: Tg4510 = F1 of [FVB-Tg(TRE-P301L 540 tau)] x [129S-Tg(Camk2a-tTA)] 1Mmay/DboJ/tau+tet genotype (2-12 mo/M and
Mouse: USF amyloid precursor protein + 630 presenilin-1 (APP+PS1) transgenic/C57BL/6J (2-24 mo; M and F)

Mouse: Tg4510 mice = F1 of [FVB-Tg(TRE- 678 P301L tau)] x [129S-Tg(Camk2a-tTA) 1Mmay/DboJ]/nontransgenic genotype (2-12 mo/M and F)

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
This is to notify you that your application to use vertebrate animals in research, testing or instruction has been approved by the Institutional Animal Care and Use Committee (IACUC).

Three months before the expiration date of your animal use form (AUF) you will be sent a reminder that your AUF will expire. If your project will extend beyond this approval period, you will have to submit a renewal for IACUC review. Please note that, according to regulations and Michigan State University policies, no significant changes may be made to your research without submitting an amendment to the IACUC for review and approval before any changes can be implemented.

All principal investigators should conduct their animal activities in accordance with the following regulations and requirements: USDA regulations (9 CFR Parts 1, 2, & 3), the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy), the Guide for the Care and Use of Laboratory Animals, 8th Edition (the Guide), and the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd Edition (Ag Guide).

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**AUF APPROVAL**
**December 20, 2017**

We are looking for your input! Please consider participating in the IACUC satisfaction survey:

https://docs.google.com/forms/d/1d9soYUP2kf40NVSMYxZK11XSjpQs1THYXAqnLBnCnQg/viewform?usp=send_form

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<td>AUF #</td>
<td>11/17-199-00</td>
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<td>PROJECT (funding):</td>
<td>Influence of Systemic Immune Inflammation upon the Tauopathy Phenotype in Mouse Models (NIH)</td>
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<tr>
<td>DATE APPROVED:</td>
<td>December 20, 2017</td>
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<tr>
<td>DATE EXPIRES:</td>
<td>December 20, 2020</td>
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</table>
MSU is registered with the United States Department of Agriculture (34-R-0017) and has an approved Animal Welfare Assurance (A3955-01) from the NIH Office of Laboratory Animal Welfare (OLAW). In addition, all components of the University are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC Unit #1047).

YOUR APPROVED AUF IS ATTACHED. IF, IN THE FUTURE, YOU NEED TO AMEND YOUR FORM, IT IS VERY IMPORTANT THAT YOU AMEND THE INFORMATION ON THIS COPY OF THE AUF AND RETURN IT TO THE IACUC OFFICE VIA EMAIL.

NOTE: If this research is DOD funded please remember that you must submit necessary approval notification to the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

Susan M. Barman, Chairperson
Institutional Animal Care and Use Committee
Michigan State University

4000 Collins Road, Room 145
Lansing, MI 48910
517.432.3154 (office) 517.432.8103
(IACUC office) barman@msu.edu