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Caitlin Rae Wessel
University of Kentucky, caitlinwessel@comcast.com

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NHE1 and Tau Pathology: Tragically Tangled Together?

Caitlin Wessel

Abstract

Alzheimer's Disease (AD) is an age-related neurodegenerative disease that poses a large public health challenge due to its complex, mixed pathology. One hallmark characteristic of AD is the development of toxic neurofibrillary tau tangles (NFT), which have been associated with the neurodegenerative component of AD. There is also compelling evidence that cerebrovascular disease puts individuals at a greater risk for experiencing the cognitive decline characteristic of dementias like AD. Past research has shown that individuals suffering from cerebrovascular disease and AD (not necessarily simultaneously) display an increased expression of the sodium-hydrogen antiporter-1 (NHE1), a plasma membrane protein ubiquitously expressed throughout the human body. To further explore this relationship between NHE1, cerebrovascular disease, and tau pathology, NHE1 knockdown and control mice were administered AAV-tau^{P301L} into the left and right ventricles via intracerebral ventricular (ICV) administration at P0/1. Pups were allowed to recover and whole brain tissue extracted after two or four weeks. Tissue was homogenized and extracted in RIPA buffer and analyzed via SDS-PAGE, followed by immunoblotting using antibodies against tau and phosphorylated tau. Identifying the role NHE1 plays in the development of tau pathology could further elucidate the mechanism relating cerebrovascular and AD tau pathology.

1. Introduction

Tau is a microtubule stabilizing protein found predominately in the axon and soma of neurons. Tau phosphorylation promotes tau association with and stabilization of the microtubules, and this process is under constant competitive regulation predominantly by protein phosphatase 2A (PP2A) and glycogen synthase kinase-3β (GSK-3β). Disruption in the regulation of tau phosphorylation (ATP depletion, mitochondrial/cellular injury, oxidative damage etc.) can lead to the hyperphosphorylation of tau protein, thus causing the proteins to dissociate from microtubules and form deleterious aggregates within the neuron.

Tau pathology is found across a wide range of diseases, two of which are Alzheimer's Disease (AD) and cerebrovascular disease (both of which are discussed in further detail in *section 2 and 3*). In fact, cerebrovascular disease puts elderly patients at a significantly greater risk for advanced cognitive dysfunction, and a majority of AD cases also comorbidly present some form of cerebrovascular disease (Saito and Ihara, 2016). Based on this comorbid expression, there is reason to believe that there is a shared mechanism between AD and cerebrovascular disease that influences the development of tau pathology.

Past research in our lab has found that animal models of diabetes and AD both display upregulation of the gene SLC9A1, which encodes Na^+/H^+ antiporter-1 (NHE1) (Song et al., 2019). NHE1 regulates intracellular pH by exchanging intracellular protons for extracellular sodium ions (Song et al., 2019). NHE1 has been clinically investigated as a therapeutic target for myocardial infarction (Fliegel, 2009), and inhibition of NHE1 has been shown to be neuroprotective against ischemic strokes (Song et al., 2019). In

relation to AD, BIN1 protein directly interacts with tau to promote tau pathology, and BIN1 also happens to be a direct ligand of NHE1 (Xue et al., 2007). NHE1 has also been found to be heavily upregulated after axonal injury, as which occurs with microtubule degradation due to tau hyperphosphorylation (Xue et al., 2007). Taken tighter, these findings hint at the potential NHE1 has as being the major player in the tau pathology development seen with AD and cerebrovascular disease.

To further elucidate the relationship between NHE1 and tau pathology, this study administered a tau pathology-inducing adeno associated virus (AAV-tau^{P301L}; exact same virus as used in Platt et al., 2016) into the brains of mice with reduced NHE1 expression and analyzed tau pathology progression two- and four-weeks post-injection. We anticipate that mice with decreased NHE1 expression should display less total tau and phosphorylated tau when compared to controls, thus showing the role NHE1 activity plays in promoting tau pathology progression.

Before going into the details of this study a brief background on AD pathology will be presented, discussing the clinical and pathological implications of the disease. Cerebrovascular disease will then be addressed in relation to AD and this study.

2. Background on Alzheimer's Disease

Around 10% of Americans over the age of 65 will develop AD, with women making up nearly 67% of this group (Santos et al., 2017). In the United States there has been a progressive increase seen in the prevalence and incidence of both AD and vascular disease (Santos et al., 2017).

AD is currently the sixth leading cause of death in the US, with cerebrovascular disease coming in one spot higher as the fifth leading cause of death in the US (**cite CDC**). In 2020, the cost of caring for AD patients will total to an estimated $305 billion, composing nearly 67% of all Medicare and Medicaid spending (**1-ad cost sheet**).

Although there is no doubt that AD places a large economic burden on the US healthcare system and is becoming a public health crisis, the pathology of this disease is quite complicated. AD is characterized by the toxic accumulation of extracellular β-amyloid peptide (Aβ) and intracellular neurofibrillary tau tangles (Hane et al., 2017). Aβ aggregation tends to precede tau aggregation (*figure i)*, but the reasons for this toxic accumulation is not well understood (Santos et al., 2017). However, Aβ has been associated with the cognitive decline seen in AD, whereas tau pathology is more closely related to the neurodegenerative component of AD (Hane et al., 2017).

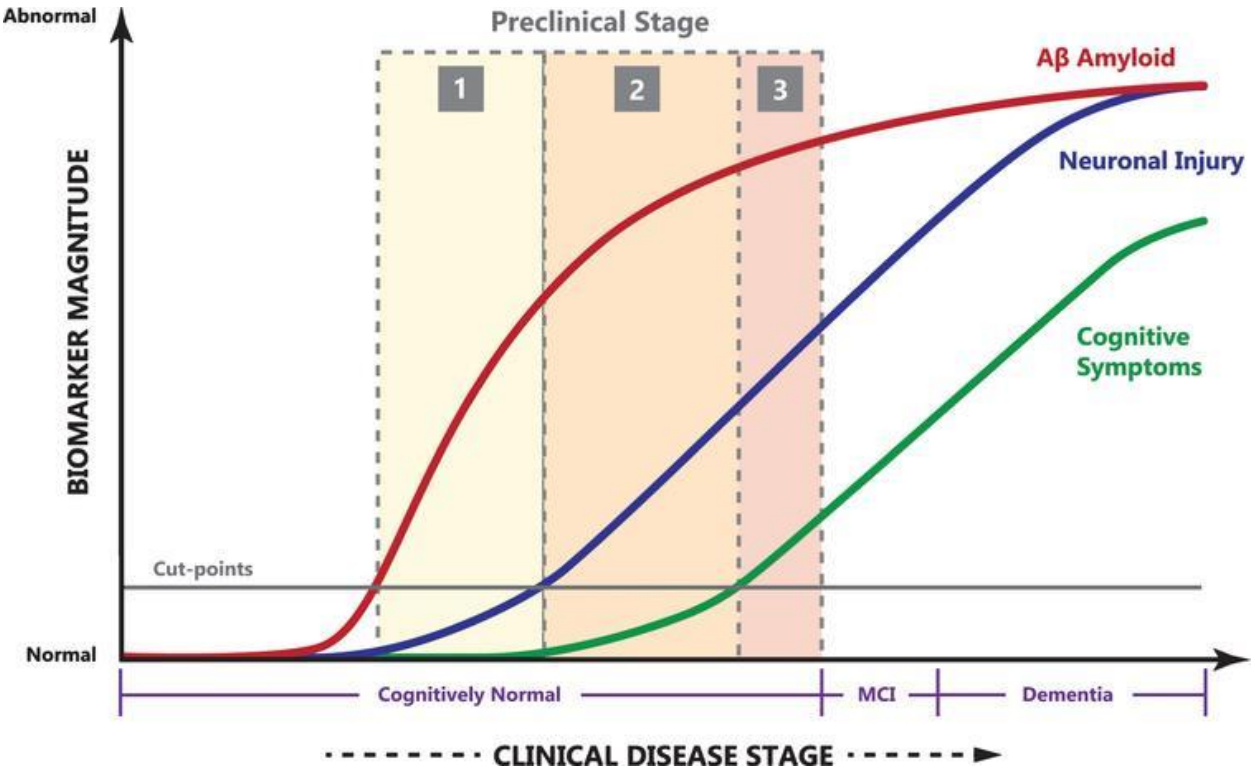


Figure i (from Hane et al., 2017). The progression of AD pathology from healthy to the preclinical phase to full blown AD pathology. Amyloid deposition precedes the development of tau pathology, which in this graph is referred to as neuronal injury. Cognitive symptoms only begin to appear after both amyloid and tau pathology progress.

Before going into detail about the proposed mechanisms that cause toxic Aβ and tau aggregation, it is important to understand how these proteins develop and function under normal physiological conditions.

Under normal conditions, amyloid peptide is cleaved from the membrane-bound protein amyloid-precursor protein (APP) by α-secretase and γ-secretase (*figure ii)* (Butterfield et al., 2013). This cleavage of APP results in the production of the non-toxic protein P3, whose role in the body is still relatively unknown (Butterfield et al., 2013). In AD cases, APP is cleaved by β-secretase and γ-secretase, thus producing an Aβ peptide instead (*figure ii;* Butterfield et al., 2013).

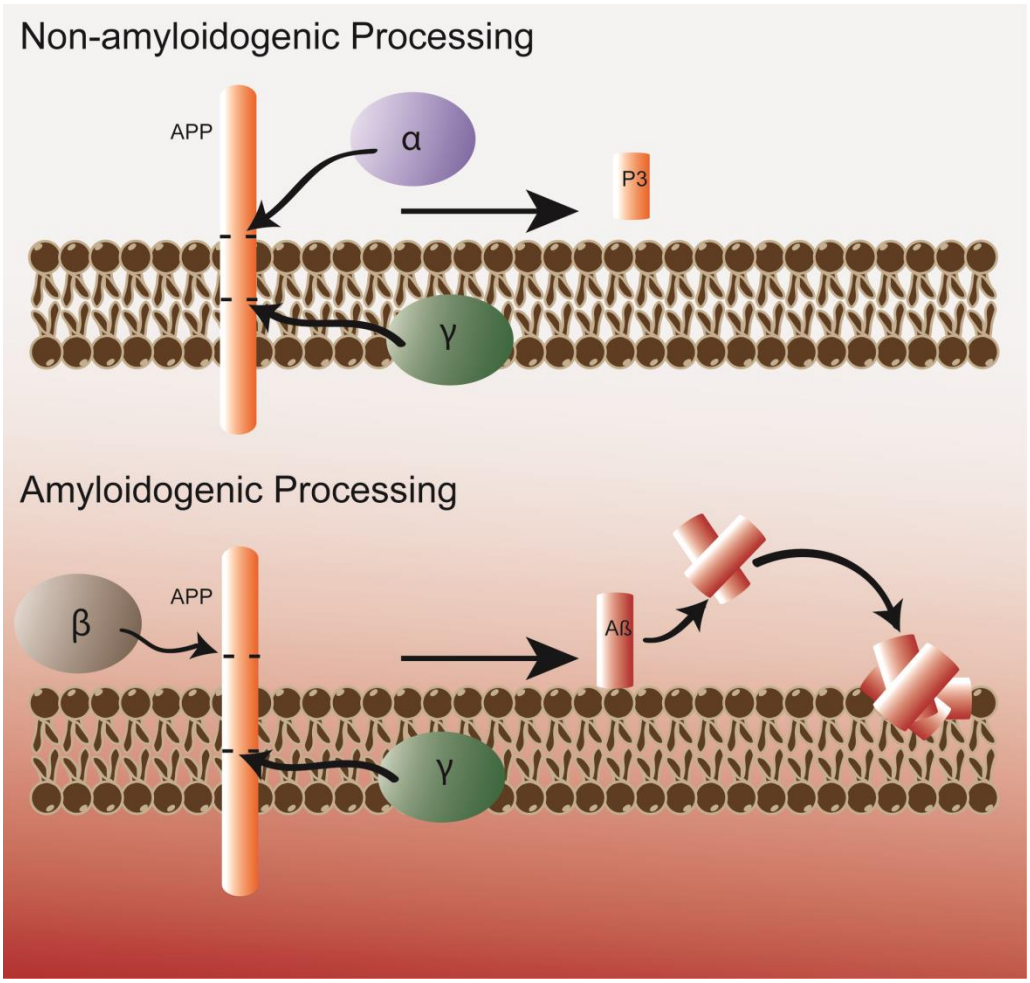


Figure ii (from Butterfield et al., 2013). APP cleavage under normal and toxic conditions. Under normal conditions, APP is cleaved by α-secretase and γ-secretase to produce non-toxic P3. In an AD brain, however, APP is cleaved by β-secretase and γ-secretase to produce toxic Aβ plaques.

Based on post-mortem human tissue, amyloid plaques composed of Aβ that is 42 amino acids long (Aβ42) tend to be more cytotoxic than aggregates predominately composed of Aβ40 (Butterfield et al., 2013). However, toxic Aβ can be produced when there is a mutation in the presenilin-1 loops of γ-secretase (Zhang et al., 2013), which then produces the same type of Aβ aggregation. Individuals can possess Aβ plaque aggregation without showing any cognitive impairment in what is known as Preclinical

AD, but these patients, if they live long enough, have an increased risk of developing AD (Dubois et al., 2015). What pushed patients to have full blown clinical AD is not just Aβ aggregation, but also pathological aggregation of tau.

Tau is a microtubule stabilizing protein found predominately in the axons and somas of neurons. Tau is the driving force behind the neurodegenerative component of AD, thus causing neurons to die and destroying neural networks (Wang et al., 2018). As a refresher, microtubules act like railroad tracks down an axon and are composed of independent α-tubulin and β-tubulin monomers held together by tau. Assembled microtubules allow for the transport of materials to and from the soma and synaptic terminal. If microtubules are like railroad tracks, then motor proteins are like railroad cars carrying cargo to its respective destination. The main motor proteins in axons are kinesin and dynein, both of which require ATP to move along the microtubule. Kinesin transports material from the soma to the synaptic terminal, whereas dynein transport material from the synaptic terminal back to the soma.

Phosphorylated tau acts like glue that holds α-tubulin and β-tubulin railroad tracks together. Tau is constantly being phosphorylated and dephosphorylated in a competitive, regulatory fashion by GSK-3β (phosphorylates tau) and PP2A (dephosphorylates tau) (Wang et al., 2018). There is another protein called PIN-1 that regulates the activity of GSK-3β (Butterfield et al., 2006), as hyperphosphorylation causes tau to fall off the microtubule and destabilized α-tubulin and β-tubulin dimers. PIN-1 is mutated in AD (Butterfield et al., 2006), resulting in a decrease in GSK-3β regulation. GSK-3β is then allowed to constantly phosphorylate tau proteins to the point of hyperphosphorylation, causing tau proteins to disassociate from microtubules (Wang

et al., 2018). These hyperphosphorylated tau proteins then form toxic neurofibrillary tangles, blocking the transport of materials along the axon (Wang et al., 2018). The destabilized microtubules fall apart, breaking down the railroad tracks for kinesin and dynein to travel along. This decrease in transport prevents new mitochondria from reaching the synaptic terminal, thus putting the neuron at an increased risk for energy depletion and oxidative damage (Butterfield et al., 2013).

One thing many researchers are able to agree on is that oxidative damage plays a significant role in the cognitive and structural changes associated with AD. Oxidative damage occurs when free radicals are able to interact with endogenous lipids and proteins, causing structural changes that alter the normal function of the organic molecule. Superoxide is one of the most common, and reactive, free radicals in the human body, and is formed during mitochondrial aerobic respiration (Radi, 2020). Mitochondria can be more prone to produce free radicals, like superoxide, under a variety of stressful conditions: high cellular energy demand, expired/old mitochondria, hypoxia, etcetera etcetera.

Under normal conditions the antioxidant superoxide dismutase (SOD) reacts with superoxide to form hydrogen peroxide, which is then broken down into water by hydrogen peroxidase (Radi, 2020). However, the free reaction between superoxide and nitric oxide, another free radical, is kinetically more favorable than the reaction between superoxide and SOD (Radi, 2020). Nitric oxide and superoxide create peroxynitrite, which is then broken down into nitrogen dioxide and a hydroxyl radical, two very reactive free radicals (Radi, 2020).

Mitochondria are under an exceptionally high risk for oxidative damage as much of the body's free radical production occurs within the mitochondria (Radi, 2020). When free radicals associate with membrane lipids, they promote the occurrence of lipid peroxidation which deteriorates the lipid bilayer, essentially punching a hole through the membrane (Radi, 2020). Lipid peroxidation of the inner mitochondrial membrane allows cytochrome C to escape from the matrix, thus being able to interact with apoptotic inducing factor (AIF). The AIF-cytochrome C complex interacts caspase 9, which then goes on to activate caspase 3 (Radi, 2020). Caspase 3 is often referred to as the executioner of the cell, initiating cellular apoptosis.

Many believe that tau aggregation causes neurodegeneration by promoting oxidative damage within mitochondria. Hyperphosphorylated tau destabilizes microtubules, thus preventing kinesin from bringing fresh mitochondria to the synaptic terminal and dynein from brining expired/damaged mitochondria back to the soma to be "recharged". This results in an accumulation of damaged mitochondria in the synaptic terminal, producing toxic levels of free radicals. These free radicals cause lipid peroxidation of mitochondrial and cellular membranes, thus leading to apoptosis. As neurons requires constant input from surrounding cells to stay alive, the death of one neuron increases the chance of death in other downstream neurons within the same neural network.

Aβ42 also promotes oxidative damage by interacting with extracellular proteins (i.e. extracellular matrix proteins) to form peptide-derived free radicals (Hensley et al., 1994). This peptide-derived free radical can also cause lipid peroxidation and the formation of 4-hydroxynonenal (HNE) (*figure iii)*. The exact role of HNE in AD is not yet totally

understood, but high cellular concentrations of HNE are associated with caspase enzyme activation and eventually cell death (Hensley et al., 1994).

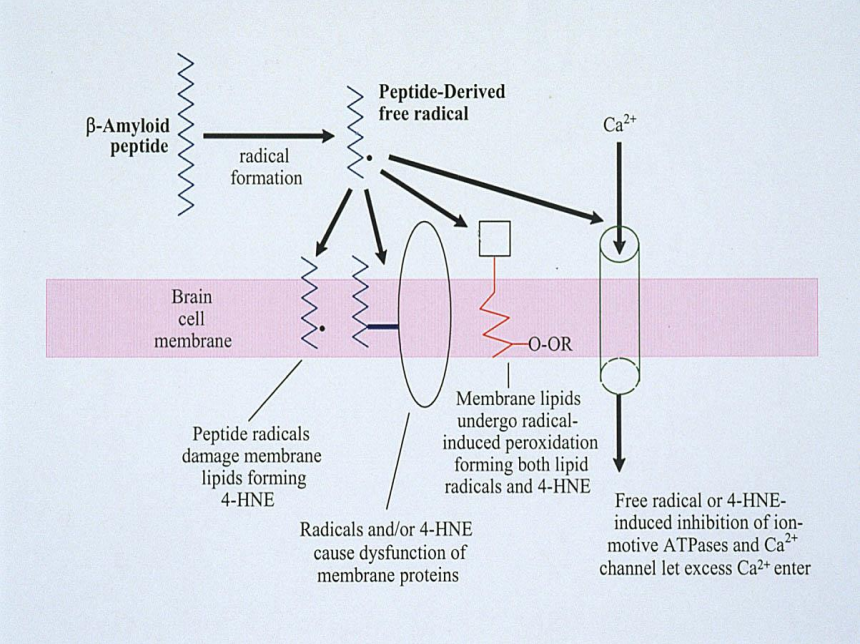


Figure iii (from Hensley et al., 1994). The relationship between Aβ-mediated radical formation, and peptide-derived free radical-mediated oxidative damage.

It is not surprising that one of the first brain regions to be affected by AD pathology is the hippocampus, as this region of the brain contains relatively low levels of endogenous antioxidants (Tome et al., 2010). Without ample levels of antioxidants to combat AD-related oxidative damage, hippocampal neurons are less likely to be able to repair oxidative damage before the initiation of apoptosis (Tome et al., 2010). As the hippocampus is crucial in the formation and retention of newer memories, neurodegeneration in this brain region produces the hallmark clinical feature of AD and other dementias: progressive memory loss.

3. Cerebrovascular Disease and AD

Cerebrovascular disease is an umbrella term that summarizes any medical condition that affects blood vessels in the brain and cerebral blood flow (Powers, 1991). For cerebrovascular diseases, vasculature in the brain is often damaged or malformed. These malformations reduce the cycling of oxygen and nutrients to the target brain regions. A common presentation of cerebrovascular disease in the clinic is ischemic stroke, and less commonly hemorrhagic strokes (Powers, 1991). Patients are at a higher risk for developing cerebrovascular disease if they have chronic hypertension, which can be caused by anything from genetics to stress to high cholesterol (Santos et al., 2017).

Cerebrovascular disease is one of the driving forces of cognitive dysfunction in the elderly (Powers, 1991). In fact, nearly 50% of dementia cases show comorbidity of vascular diseases (Habeych et al., 2015), thus raising questions about how these two deadly diseases are potentially related.

There are many overlapping risk factors between AD and cerebrovascular disease. From a genetics standpoint, studies have shown that possession of just one ε4 allele for the apolipoprotein E (APOE) gene is a risk factor for both AD and cerebrovascular disease (Santos et al., 2017). Advancements in healthcare are allowing people to live longer, and in aged 85yrs and older are the fastest growing portion of the US population (Santos et al., 2017**).** As the elderly population is continuing to grow, we are seeing an increased incidence of both AD and cerebrovascular disease within this population, indicating age as a risk factor for these two diseases (Santos et al., 2017).

From a lifestyle standpoint, people older in age tend to be less active and have less variety in their diet (Santos et al., 2017).

These common lifestyle habits put individuals at a higher risk of hypertension and high cholesterol. As mentioned earlier hypertension is a risk factor for cerebrovascular disease, as the delicate blood vessel in the brain are more prone to damage (Santos et al., 2017). Damaged cerebral vasculature would alter the amount of oxygen able to reach brain tissue, thus putting neurons at a significantly greater risk of oxidative damage due to mitochondrial stress. High cholesterol can block arteries and veins, thus causing strokes if the blockage occurs in the brain or medial/anterior cerebral arteries (Santos et al., 2017). Strokes prevent blood from reaching target regions of the brain. This depletion in nutrients and oxygen would provide stressful conditions for neurons in the effected tissue, thus increasing the risk of oxidative damage (Santos et al., 2017). As discussed in *section 2*, oxidative damage eventually initiates cell death via activation of caspase enzymes. This relationship between cerebrovascular disease risk factors and oxidative damage may provide a clue as to how this disease is related to AD.

Another risk factor for cerebrovascular disease is diabetes mellitus (from here on referred to as diabetes), and research has shown that many diabetes patients go on to develop AD as well (Santos et al., 2017). However, the mechanism behind how insulin inefficiency puts patients at a greater risk for cognitive impairment remains relatively unknown. The literature on this relationship seems to be broken up into two schools of thought. One side believes that it is the excessive levels of glucose and insulin in the blood that leads to AD, since even non-diabetic AD patients have significantly higher levels of insulin and blood-glucose than healthy controls (Santos et al., 2017). The other

group believes that insulin deficiency, due to diabetes-related insulin resistance, causes AD by preventing insulin from prompting normal glucose metabolism in the brain (Santos et al., 2017). Impaired glucose metabolism leads to energy depletion and, as discussed previously, promotes the production of free radicals and oxidative damage within affected neurons.

Regardless of the mechanism causing diabetes to be a risk factor for AD, the relationship between the two diseases undebatable. As such, this study uses a mouse model of AD that incorporates diabetes pathology as the control strain. In an attempt to elucidate the role NHE1 plays in the development of characteristic AD tau pathology and cerebrovascular disease, it seems appropriate to use a control mouse strain that incorporates a risk factor for both AD and cerebrovascular disease.

4. Methods

Two strains of mice were used in this study: db/AD mice served as control with normal NHE1 expression, and NHE1 knockdown mice served as the experimental group with reduced NHE1 expression. Pups at postnatal day 0 or 1 (P0 or P1, respectively) were cryoanesthetized for three minutes prior to intracerebral ventricular (ICV) injections. Based on the procedure outlined in Kim et al., 2013[5], 2μl of either AAV-GFP or AAV-tau^{P301L} was administered into the left and right ventricles using a Hamilton syringe (Hamilton, 80301) and 32-gauge needle. Care was taken to avoid excessive freeze-thawing of the viruses. After ICV injections, pups were placed on a heating pad underneath a warming lamp until they regained normal color and movement and were

then immediately returned to the mother. Health checks were conducted every day for the first 5 days post-injection, and then once a week for the remainder of the study.

Mice were euthanized at P14 or P28 via intraperitoneal administration of Euthasol (100mg/kg). Brain hemisphere were removed and immediately frozen at -80°C. The next day the left hemisphere was homogenized in 1ml/200mg RIPA buffer and centrifuged at 100,000 x *g* for one hour. Supernatant was removed and a BCA assay was run before preparing samples for western blot analysis.

Western Blots were run on 4-12% Bis-Tris gel (Criterion XT Precast Gel, BioRad), with 30ug of protein loaded into each well. Gels were run at 120V for 1.5hrs and transferred onto a nitrocellulose membrane (ThermoFisher) at 1amp for 1hr. Membranes were then blocked overnight at 20°C in a 5% milk solution. The membrane was exposed to the primary antibody for 1hr and to the secondary antibody for 45mins. The primary antibody probed for phosphorylated tau (pSer396, Invitrogen) and was diluted 1:1000 in a 5% milk solution, while the goat anti-rabbit secondary antibody (Fisher Scientific) was diluted 1:5000 in a 1% milk solution. West dura signal substrate (SuperSignal West Dura Substrate; Thermo Scientific) was placed on the membrane for 5mins, and then membranes were exposed on film paper (Blue Basic Autorad Film 8x10; BioExpress) for 1min.

Tissue samples extracted in RIPA buffer were also run through commercial tau ELISAs for further analysis. Phosphorylated tau was measured using the Human Tau [pT181] phosphoELISA Kit (ThermoFisher Scientific), in which samples were diluted 1:5 in standard diluent buffer (Human Tau [pT181] phosphoELISA Kit, TheroFisher Scientific). Total tau was measured using the Human Tau [total] ELISA Kit

(ThermoFisher Scientific), in which samples were also diluted 1:5 in standard diluent buffer (Human Tau [total] phosphoELISA Kit, TheroFisher Scientific). All qualitative results were corrected based on relative dilution before statistical analysis.

Values are represented as *mean±standard error of the mean (SEM),* and statistical analysis was conducted via Two-Way ANOVA with a p value of *p<0.05.*

5. Results

No difference was found in total tau ELISA concentrations after AAV-tau^{P301L} administration between dbAD (769.29±42.07pg/ml) and NHE1 (669.17±28.22pg/ml) strains (mean±SEM, p>0.05). However, there was also no significant difference in total tau concentrations in dbAD mice between the AAV-GFP (830.97±38.90pg/ml) and AAV-tau^{301L} (769.29±42.07pg/ml) administration groups (mean±SEM, p>0.05).

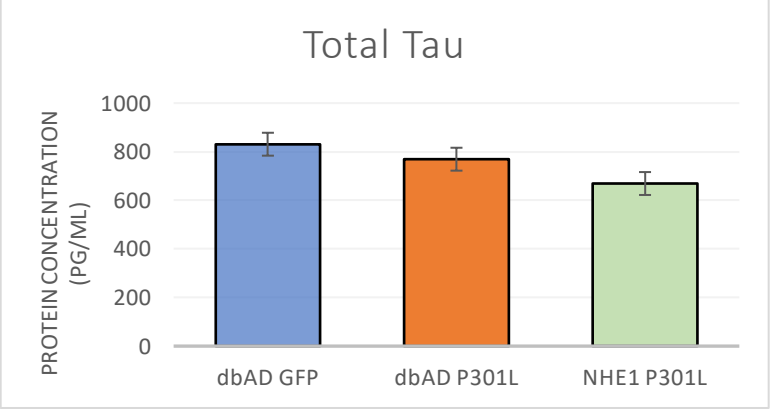


Figure 1. Total tau concentration (pg/ml) between dbAD mice administered ICV AAV-GFP (dbAD GFP), dbAD mice administered ICV AAV-tau^{P301L} (dbAD P301L), and NHE1 knockdown mice administered ICV AAV-tau^{P301L} (NHE1 P301L). No difference was found between any of the groups (p>0.05).

The degree of phosphorylated tau aggregation was much lower than total tau, as is generally expected. However, no difference in phosphorylated tau was found between AAV-tau^{P301L}-injected dbAD (18.28±2.09pg/ml) and NHE1 (18.99±1.82pg/ml) strains (*figure 2;* mean±SEM, p>0.05). As seen with the total tau ELISA, there was no significant difference in phosphorylated tau concentrations in dbAD mice between the AAV-GFP (24.95±2.15pg/ml) and AAV-tau^{301L} (18.28±2.09pg/ml) administration groups (*figure 2;* mean±SEM, p>0.05).

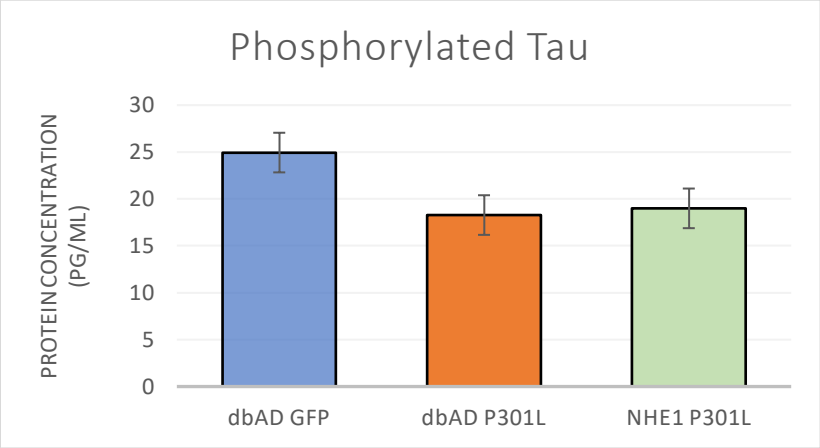
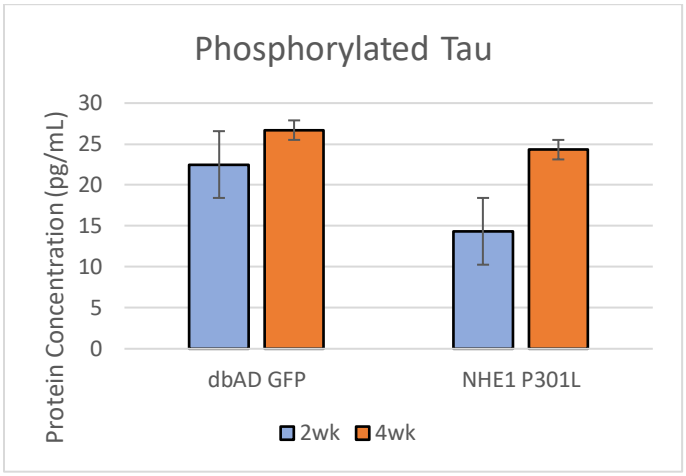


Figure 2. Phosphorylated tau concentration (pg/ml) between dbAD mice administered ICV AAV-GFP (dbAD GFP), dbAD mice administered ICV AAV-tau^{P301L} (dbAD P301L), and NHE1 knockdown mice administered ICV AAV-tau^{P301L} (NHE1 P301L).

The only significant age group effect seen was between P14 (14.34±1.01pg/ml) and P21 (24.21±2.53pg/ml) levels of phosphorylated tau in NHE1 pups administered AAV-tau^{P301L} (*figure 3a;* mean±SEM, p>0.05). This trend is likely due to a larger brain size than to tau pathology development, as a similar trend is seen total tau between the

2wk (22.49±3.70pg/ml) and 4wk (26.69±2.62pg/ml) dbAD mice administered AAV-GFP (*figure 3b;* mean±SEM, p>0.05).

A.



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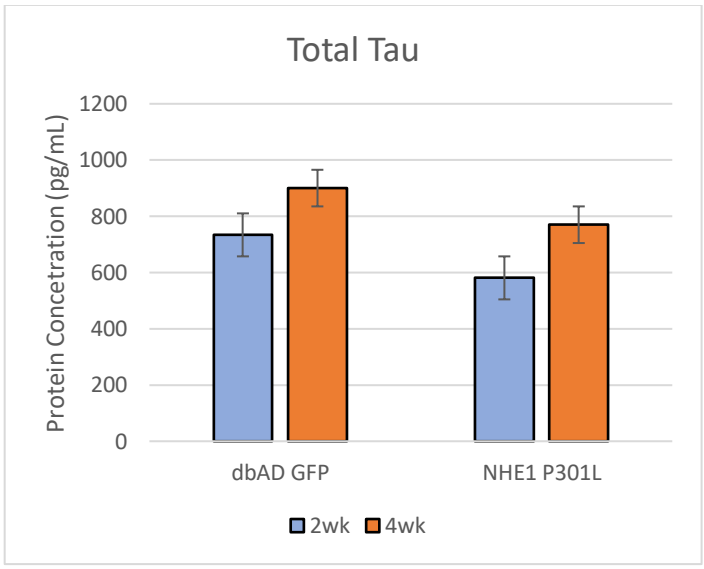


Figure 3. Comparison in tau pathology between 2wk and 4wk old mice. A) Differences in phosphorylated tau, and B) differences in total tau concentrations (pg/ml between dbAD mice administered ICV AAV-tau^{P301L} (dbAD P301L), and NHE1 knockdown mice administered ICV AAV-tau^{P301L} (NHE1 P301L).

There was no significant in the ratio of phosphorylated tau to total tau (phosphorylated tau/total tau x 100) between the dbAD GFP (2.99±0.20), dbAD P301L (2.36±0.22), and NHE1 P301L (2.77±0.17) groups (*figure 4;* mean±SEM, p>0.05).

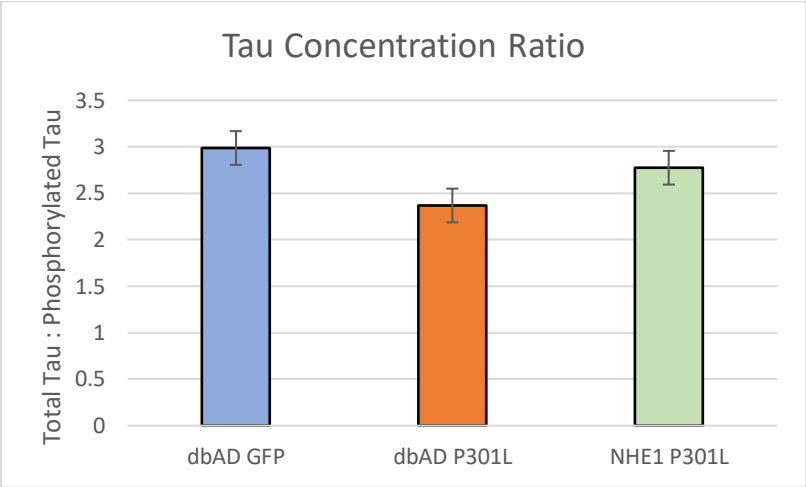
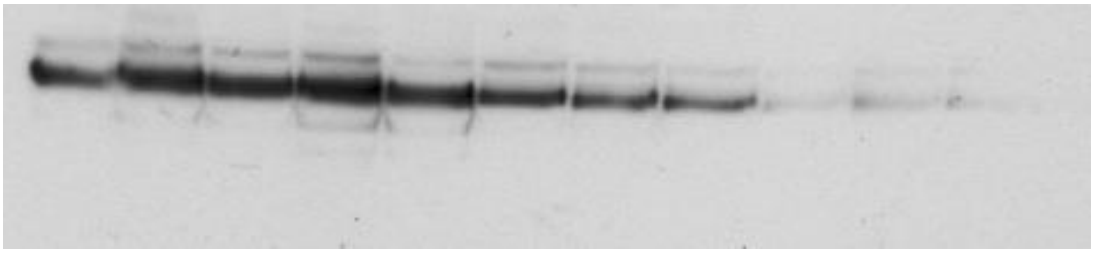


Figure 4. Ratio between phosphorylated tau and total tau (phosphorylated tau/total tau X 100).

Western blot analysis, however, did show some differences in the molecular weight of phosphor-tau (pSer396 probed) between groups. dbAD AAV-GFP samples showed phosphotau bands at 50kDa (*figure 5a*), whereas all samples administered AAV-tau^{P301L} showed 29kDa banding (*figure 5b-c*). NHE1 AAV-tau^{P301L} samples showed weak 50kDa banding but strong 20kDa banding (*figure 5c*), whereas dbAD AAV-tau^{P301L} showed strong banding at 50kDa and 20kDa (*figure 5b)*.

A. 

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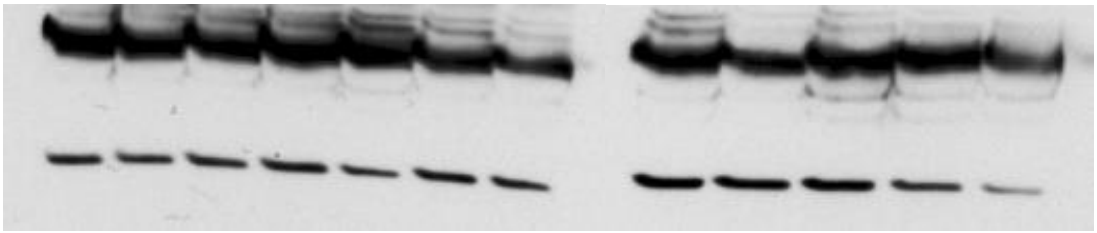

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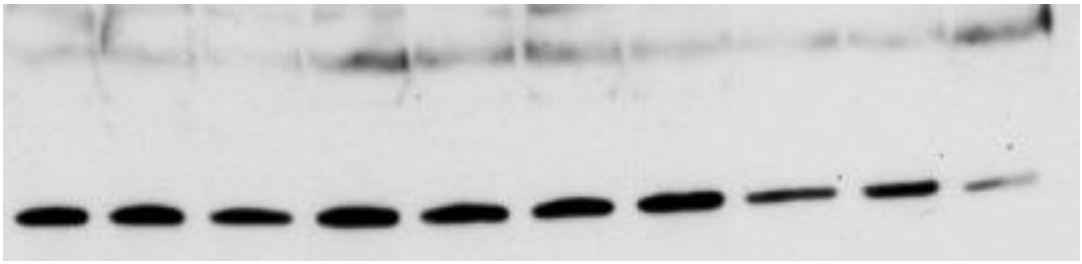

Figure 5. pSer396 probed western blot of A) dbAD GFP, B) dbAD P301L, and C) NHE1 P301L. AAV-GFP administration in dbAD mice only produced a band of 50kDa phosphotau, whereas AAV-tau^{P301L} administration in both dbAD and NHE1 strains produced a phosphotau band at 20kDa and 50kDa. However, NHE1 AAV-tau^{P301L} samples displayed a weak 50kDa phosphotau band whereas dbAD AAV-tau^{P301L} samples displayed a strong 50kDa phosphotau band.

6. Discussion

This study sought to elucidate a relationship between NHE1 expression and the development of tau pathology. After ICV administration of AAV-tau^{P301L}, NHE1 knockdown mice were anticipated to possess lower total tau and phosphorylated tau concentrations than dbAD control mice. The results of this study found no difference between NHE1 and dbAD strain development of tau pathology after AAV-tau^{P301L} administration. There was also no difference between groups in the ratio of phosphorylated tau to total tau. Taken together these results reject our initial hypothesis, indicating that NHE1 knockdown does not affect the development of tau pathology.

The AAV-tau^{P301L} virus used in this study had been stored at -80°C for a couple years prior to our use, and consequently may have lost viral potency. This potential reduction in AAV potency would have insufficiently produced tau pathology within the brain, thus producing the perceived lack of effect seen in our study. This study should be conducted again using a brand-new virus in order to eliminate this confound. We know the virus sufficiently entered the brain as tissue from the AAV-GFP group was tinted a shade of greenish-yellow, indicating that our ICV protocol allowed for sufficient viral infection in the brain.

However, western blot analysis showed difference in the molecular weight of aggregated phosphorylated tau between dbAD and NHE1 knockdown strains administered ICV AAV-tau^{P301L}. NHE1 samples had relatively fewer 50kDa phosphate aggregates compared to dbAD samples, which had robust levels of both 50kDa and 20kDa aggregates. These results suggest that, although the overall levels of phosphorylated tau might be the same between NHE1 and dbAD strains after AAV-tau^{P301L}, tau pathology in NHE1 knockdown strains is composed of low molecular weight tau aggregates.

More focus should be placed on investigated the role NHE1 expression plays in the molecular weight of tau aggregation, and future studies should also explore the effects NHE1 expression has on the long-term development of tau pathology by including longer timepoints.

Acknowledgements

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