A NOVEL TAUOPATHY MODEL UTILIZING *DICTYOSTELIUM DISCOIDEUM*

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ABSTRACT

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by Kristi Miller

Alzheimer's disease is a chronic, progressive brain disorder, affecting approximately 35 million people worldwide. Pathologically, Alzheimer's disease is characterized by the accumulation of two types of brain lesions: senile plaques and neurofibrillary tangles. Neurofibrillary tangles are found within neurons and are formed by the aggregation of paired helical filaments, in which the main component is tau, a microtubule associated protein. The mechanisms by which tau aggregates into filaments remains uncertain, but studies have shown that phosphorylation and proteolysis by calpain are major contributors to tau-mediated toxicity. Cleavage of tau by calpain is thought to result in a highly toxic form of tau called the 17kD fragment. In this study, we are using *Dictyostelium discoideum* as a novel tauopathy model to investigate how these post-translational modifications of tau lead to its cellular toxicity. *Dictyostelium* cells were transformed with plasmids to express wild-type (tau^{WT}) and mutant forms of human tau (phosphorylation-incompetent, tau^{AP}; calpain-resistant, tau^{CR}; tau 17kD fragment, tau^{17kD}). The constitutive expression of tau^{WT}, tau^{CR}, and tau^{17} was sufficiently toxic to induce cell death. However, tau^{CR} expression appeared to be slightly less toxic than expression of tau^{WT} or tau^{17}. Expression of tau^{AP} was not toxic to *Dictyostelium* wild-type cells. These results suggest that phosphorylation plays an important role in tau toxicity. Because the expression of tau under a constitutive promoter rapidly induces cell death, we are now using an inducible vector system in which transcription of the tau gene can be reversibly turned on with the addition of doxycycline. Thus far, tau^{WT} and tau^{AP} has been successfully cloned into the inducible vector.

Once all of the inducible vector plasmids are constructed, we will characterize the effect of tau and mutant tau expression on *Dictyostelium* survivability and cell division. Development of *Dictyostelium* as a tauopathy model may provide a unique system for high-throughput screening of new therapeutic molecules for the treatment of Alzheimer's disease.

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CHAPTER I

INTRODUCTION

Alzheimer's Disease

Alzheimer's disease is a chronic, progressive brain disorder, affecting approximately 35 million people worldwide. In the United States, Alzheimer's disease is the 6th leading cause of death for those over the age of 65 with nearly 5.5 million Americans affected (Herbert *et al.* 2005; Koffie *et al*. 2011). Memory loss is often the first recognizable sign of Alzheimer's disease with subsequent loss of motor skills, decreased sensory perception, and an inability to use or understand language. Pathologically, Alzheimer's disease is characterized by the accumulation of two types of brain lesions: senile plaques and neurofibrillary tangles. Senile plaques are found within the extracellular space of the brain and are caused by the accumulation of the β-amyloid peptide, which is derived from the amyloid precursor protein (Buee *et al*. 2000; Koffie *et al*. 2011). Neurofibrillary tangles are found within neurons and are formed by the aggregation of paired helical filaments, in which the main component is tau, a microtubule associated protein. Any neurodegenerative disease in which tau protein aggregates abnormally in the brain, including Alzheimer's, is considered to be a tauopathy (Buee *et al*. 2000).

Tau

Tau proteins are found within the central nervous system and normally function to promote the assembly and stability of microtubules through interactions with tubulin (Buee *et al*. 2000). There are six distinct isoforms of tau found within the human brain, which are generated by the alternative splicing of tau mRNA. Developmental expression is varied for each tau isoform, indicating that they may each have discrete physiological roles (Goedert and Jakes 1990). The amino-terminus of the tau protein, also known as the projection domain, allows tau to

interact with the neural plasma membrane and cytoskeletal elements. The carboxyl-terminus of the tau protein contains a microtubule repeat domain, which allows for the binding of tau to microtubules to promote their polymerization and stabilization (Buee *et al*. 2000; Buee *et al.* 2010). In a healthy adult human, tau is found in the axons of nerve cells. However, upon examination of an adult brain affected with Alzheimer's disease or other tauopathy, tau is found in the cell body and dendrites of nerve cells. The normally soluble protein tau becomes insoluble and aggregates to form paired helical filaments (Goedert 2004). The abnormal aggregation of tau has been proposed to cause dendritic amputation, impaired synaptic transmission, generalized neuronal dysfunction, and ultimately neurodegeneration (Kremer *et al.* 2011). The mechanisms by which tau aggregates into filaments remains uncertain but studies have shown that phosphorylation may be an essential mediator (Buee *et al.* 2010; Steinhilb *et al.* 2007).

The most common post-translational modification of tau is phosphorylation. The tau protein contains 85 potential phosphorylation sites and 71 of these sites have been linked to tau pathology (Buee *et al*. 2010). Phosphorylation of tau can lead to a decrease in its ability to stabilize and polymerize microtubules thereby reducing neuronal cytoskeleton stability (Martin *et al*. 2011). The various kinases that are involved in tau phosphorylation can be divided into two main groups. The first are proline-directed protein kinases, which phosphorylate tau at serine or threonine sites that are followed by proline. These kinases include: glycogen synthase kinase 3 (GSK3), mitogen activated protein kinase (MAP), tau-tubulin kinase, and cyclin-dependent kinases, cdc2 and cdk5 (Buee *et al*. 2000; Steinhilb *et al.* 2007). The second group of kinases that phosphorylate tau are non-proline-directed kinases which include cyclic-AMP-dependent kinase (PKA), calcium/calmodulin-dependent protein kinase II (CaMPK II), and microtubule-affinity regulating kinase (MARK) (Buee *et al*. 2000). Some phosphorylation is required for the

normal function of tau but it is the excessive phosphorylation of tau by proline directed kinases that is thought to lead to toxicity. AD-affected tissue has been used to develop disease-associated antibodies and many of these antibodies recognize phosphorylated serine/proline and threonine/proline sites (sites which are not phosphorylated in age-matched, non-dementia adults), suggesting these sites may be particularly important in disease (Buee *et al*. 2000; Steinhilb *et al.* 2007).

Calpain

The protease calpain has been shown to cleave tau, thereby creating highly toxic tau fragments. Park and Ferreira (2005) used hippocampal neurons from rat embryos treated with pre-aggregated β-amyloid and tau to examine the potential role of tau proteolysis in β-amyloid-induced neurodegeneration. They found that hippocampal neurons treated with pre-aggregated β-amyloid without tau did not exhibit signs of degeneration. However, hippocampal neurons treated with pre-aggregated β-amyloid and tau resulted in degeneration. The 17kD fragment was detected in degenerating neurons and pretreatment of hippocampal neurons with calpain inhibitors blocked the formation of the 17kD fragment. This research provides evidence that calpain does in fact cleave tau, generating a highly toxic 17kD fragment. It is hypothesized that calpain cleavage and generation of the 17kD tau fragment induces toxicity because of a decrease in full-length tau to stabilize microtubules. Microtubules are important for the transport of essential nutrients to the distal ends of neurons. In the absence of full-length tau, microtubule stability is reduced. This could lead to microtubule depolymerization thereby promoting the disruption of the entire microtubule network and creating neural degeneration (Park and Ferreira 2005).

Drosophila Melanogaster as a Tauopathy Model System

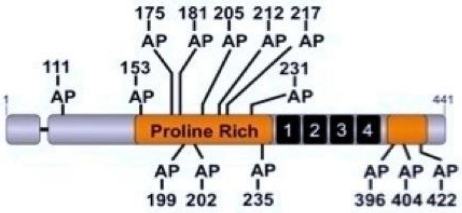
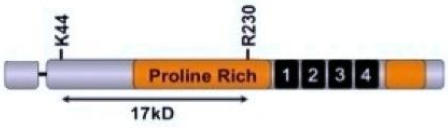
Drosophila has proven to be an exceptional model to study tau pathology in neurodegenerative disease. This model organism is highly amenable to molecular and genetic analysis. Its genome encodes approximately 13,000 genes compared to its human counterpart of 27,000 genes. Additionally, *Drosophila* has a short generation time, has high fecundity, and is inexpensive to use and manipulate in the laboratory. Researchers have utilized the fly model by expressing wild type and mutant forms of human tau in the fly eye (Hirth 2010; Reinecke *et al.* 2011; Steinhilb *et al.* 2007; Wittmann 2001). Toxicity in the fly eye produces a phenotype that is exceptionally discernable. Expression of human tau in the fly eye retina leads to a "rough eye phenotype", which can be characterized by a reduction in the size of the eye as well as a disruption of the regular array of bristles and lenses. Another benefit to using the fly eye model is the ability to perform pharmacologic and genetic screens for enhancers and suppressors of tau toxicity (Shulman and Feany 2003; Muqit and Feany 2002).

The Steinhilb lab has utilized *Drosophila* as a tauopathy model to determine the effects of proline-directed kinases on tau toxicity. Steinhilb (2007) and colleagues created a tau construct deemed tau^{AP} containing fourteen point mutations from serine or threonine to alanine (Table 1). These point mutations specifically insure that all 14 of the disease associated serine or threonine sites directly followed by proline cannot be phosphorylated on tau. *Drosophila* fly eyes expressing tau^{WT} were found to have extensive toxicity while *Drosophila* expressing tau^{AP} showed no toxicity. These results suggest that SP/TP phosphorylation is essential for the toxicity of tau (Steinhilb *et al.* 2007).

In order to further elucidate the mechanisms by which tau toxicity occurs, additional research using the fly model was conducted to determine the relationship between

phosphorylation and cleavage of tau to produce the highly toxic 17kD fragment. Reinecke and colleagues (2011) utilized calpain mutants and created a calpain-resistant form of tau (tau^{CR}) in order to test their hypothesis that calpain cleavage increases toxicity. No toxicity was observed in fly eyes expressing tau^{CR} compared to flies expressing tau^{WT}. They also created an additional tau mutant expressing a 17KD fragment, tau^{17}, to evaluate the toxic effects of this fragment (Table 1). While they were unable to highly express tau^{17}, substantial toxicity was found in the fly eye with only moderate expression. Phosphatase assays were performed in order to determine the degree of phosphorylation on the proteins produced from tau^{CR} and tau^{17} flies. Results revealed that tau^{CR} was phosphorylated to the same degree as full-length wild type tau *in vivo*. The tau^{WT} and tau^{CR} samples were also analyzed for the presence or absence of the 17kD fragment. It was expected that the 17kD fragment would not be found in the tau^{CR} sample because of the mutations present in this form of tau making it calpain resistant, and thereby unable to be cleaved into the 17kD fragment. The 17kD fragment was expected to be detected in the full-length tau^{WT} sample. However, the 17kD fragment not detected in the tau^{WT} or tau^{CR} sample. It was hypothesized that this may have been due to a high degradation rate of the 17kD fragment in the fly eye or the inability to express high levels of recombinant tau protein (Reinecke *et al.* 2011). The use of a different model organism in these experiments may enable the expression of higher levels of recombinant tau^{WT} and reveal if phosphorylation regulates the cleavage of tau and the production of the 17kD fragment.

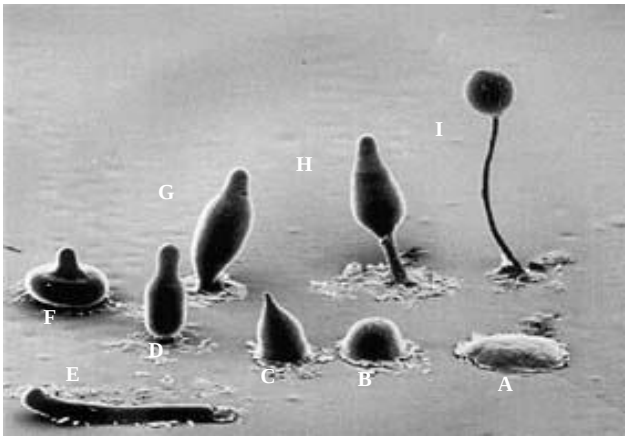
Table 1. Diagram of wild-type human tau and various mutant forms of tau created.

tau^{WT}  Wild-type tau found in the human brain. Composed of a proline rich region and microtubule repeat domain.	tau^{CR}  Mutated lysine at site 44 to glutamine and arginine at site 230 to glutamate thereby facilitating calpain resistance
tau^{AP}  Contains 14 point mutations from serine or threonine to alanine located on either side of a proline region in order to make these sites phosphorylation incompetent	tau^{17}  Only expresses the region of tau composed of amino acid 44-230 which make up the toxic 17kDa fragment.

Dictyostelium as a Model Organism

Dictyostelium discoideum is a cellular slime mold that provides a simple eukaryotic system for the study of protein function. In the vegetative state, *Dictyostelium* exist as unicellular, soil dwelling organisms that feed on bacteria. When starvation occurs the multicellular stage of their life cycle is initiated and individual haploid amoebae aggregate together to form a multicellular mass. The precursors of the stalk and spore cells, prestalk and prespore cells, are first detected during the end of aggregation when cells have grouped together to form a mound. If environmental conditions do not favor fruiting body formation, the mound develops into a migratory slug (Early 1999, Figure 1). By the slug stage a distinct pattern of prestalk and prespore cells is produced, with the tip composed solely of prestalk cells. The remainder of the

slug is composed of prespore cells and prestalkO/ALC's (anterior like cells) (Figure 2). A ratio of 1:4 prestalk to prespore cells is maintained in the slug (Mohanty and Firtel 1999).



A = loose aggregate
B = tight mound
C = tipped aggregate
D = first finger
E = migratory slug
F = Mexican hat
G = early pre-culminant
H = later pre-culminant
I = culminant/fruiting body
(Scanning EM image by Mark Grimson & Larry Blanton, Texas Tech University)

Figure 1. *Dictyostelium discoideum* life cycle.

Cells maintain the capacity to transdifferentiate until culmination, when cells become committed to being either stalk or spore cells. Culmination is triggered by light or a decrease in ammonia concentration. The fruiting body formed is composed of three main elements: a large spore head, a stalk that supports the spore head, and a basal disc that anchors the stalk to the substratum. Each element is derived from specific precursor cells: spores from prespore, the stalk from prestalkA and prestalkO cells, and the basal disc from prestalkB cells (Early 1999). This rapid development, which normally concludes around 24 hours, along with its distinct cell pattern, evolutionarily conserved signaling pathways, and amenability to molecular genetic analysis makes *Dictyostelium* a highly effective model organism (Urushihara 2008).

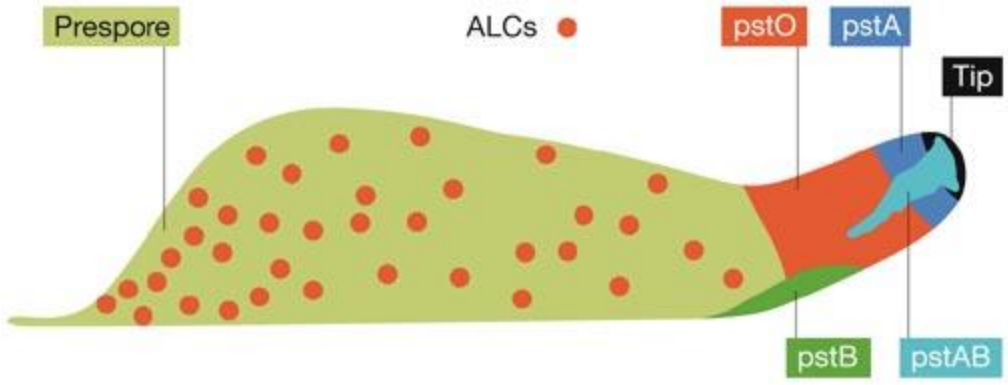


Figure 2. Distribution of cell types in a *Dictyostelium discoideum* slug (Williams 2006). The prestalk region contains prestalkA (*pstA*) and prestalkO (*pstO*) cells, as well as prestalkB (*pstB*). Cell names come from expression pattern of cell-type specific genes. PstA and pstO express *ecmA*, pstB expresses *ecmB*, and pstAB expresses *ecmA* and *ecmB*. The anterior-like cells (ALCs) comprise roughly 10% of cells that stain in the posterior region.

Prior Studies: Dictyostelium as a Model for Neurodegenerative Disease

Dictyostelium has been effectively utilized in research studies on neurodegenerative disease. Reyes *et al.* (2009) used *Dictyostelium* as a model to study the formation of Hirano bodies, which are cytoplasmic inclusions composed of actin and actin-associated protein aggregates that have been found in brains affected with Pick's and Alzheimer's disease. The authors used *Dictyostelium* cells expressing a carboxy-terminal fragment of the 34 kDa calcium sensitive actin binding protein (ABP34) fused to GFP under the control of both a constitutive and inducible promoter to test their hypothesis that Hirano bodies are formed by aggregation of many small inclusions (Reyes *et al.* 2009). *Dictyostelium* cell motility along with the use of an inducible promoter system were essential in allowing the authors to view and characterize the formation of Hirano bodies in real time.

Another study conducted by McMains and colleagues (2010) used *Dictyostelium discoideum* as a model organism to study the molecular mechanisms involved in the processing of human amyloid precursor protein (APP) by the presenilin/γ-secretase complex. While

Dictyostelium does not contain an ortholog to human APP, a bioinformatics search revealed that *Dictyostelium* contains orthologs of the human subunits of the presenilin/γ-secretase complex. The authors created single and double mutants of the presenilin/γ-secretase complex components and compared their ability to process human APP in wild-type cells. Phenotypic assays were performed on mutant cells and revealed that the human APP could be processed to form amyloid peptides by the *Dictyostelium* orthogs of the presenilin/γ-secretase complex (McMains *et al.* 2010). The presence of human gene orthologs in addition to *Dictyostelium's* unique and rapid development enabled the authors to characterize the presenilin/γ-secretase complex.

Dictyostelium as a Tauopathy Model

Due to the conservation of many cellular and developmental processes across species, the study of *Dictyostelium* may aid in the understanding of more complex organisms. *Dictyostelium* can be cultivated easily in the lab and is amenable to otherwise difficult molecular and genetic experimental techniques. The life cycle of *Dictyostelium*, which concludes around 24 hours, allows for the viewing of all stages of development in real time. All of these aspects will enable the use of *Dictyostelium* as a tauopathy model. It is especially important to note that *Dictyostelium* contains orthologs of human genes for kinases, phosphatases, and proteases known to be involved in tau toxicity. Orthologs of human proline-directed protein kinases, which phosphorylate tau at serine or threonine sites that are followed by proline, such as glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase (cdk) can be found in *Dictyostelium* (Buee *et al.* 2000; Ferkey and Kimelman 2000). An ortholog of calpain, the protease suspected of tau cleavage to form the toxic 17kD fragment, has also been discovered within *Dictyostelium* (Huang *et al.* 2002*)*. It is advantageous that these orthologs are present because it will allow us to view the modification of tau within *Dictyostelium*. Also, we can view the modification of tau in

real time due to the short developmental life cycle of *Dictyostelium* (Early 1999). There is not a human tau ortholog present within *Dictyostelium* and this is beneficial, because then we can be certain that there is no endogenous tau interfering with the human tau in experiments (dictybase.org). Additionally, the convenience of pre-constructed *Dictyostelium* mutants that can be ordered from Dictybase will allow for a broad range of characterization of tau in the presence or absence of various kinases (Table 2).

Table 2. *Dictyostelium* mutants available from Dictybase.org and their accession information.

Dictyostelium mutant description	Glycogen Synthase Kinase (GSK) null (*gsk*$^-$)	Cyclin dependent kinase overexpressor (*cdk+*)	PKA catalytic subunit null mutant (*pkaC*$^-$)	PKA catalytic subunit overexpressor (*pkaC*$^+$)
Strain ID	DBS0236268	DBS0237098	DBS0236783	DBS0236484
Gene ID	gskA	cdk5	pkaC	pkaC
Dictyostelium mutant description	Serine protease null (*tagA*$^-$)	Serine protease null (*tagB*$^-$)	Serine protease null (*tagC*$^-$)	E3 ubiquitin ligase component FbxA null (*fbxA*$^-$)
Strain ID	AK859	AK228	DBS0236975	DBS0235953
Gene ID	tagA	tagB	tagC	fbxA

Hypothesis and Experimental Aims

Using *Dictyostelium as a* tauopathy model may provide new insights into tau's role in neurodegeneration. Hyperphosphorylation and the cleavage of tau to produce the 17kD fragment have been associated with tau toxicity, but the relationship between these two post-translational modifications remains unclear (Park and Ferreira 2005). Efforts to detect the 17kD fragment in recombinant tau protein samples *in vitro* remain unsuccessful. It is uncertain whether the 17kD fragment is being produced or if it is being degraded quickly in these samples (Steinhilb *et al.*

2007; Reinecke *et al.* 2011). Using *Dictyostelium* as a novel tauopathy model may enable high expression of human and mutant tau for biochemical analysis and allow us to answer the question: Does phosphorylation regulate the cleavage of tau and the production of the 17kD fragment?

In this study, we were able to constitutively express human and mutant forms of tau in *Dictyostelium* cells. Expression of tau^{WT}, tau^{CR}, and tau^{17} was sufficiently toxic to induce cell death. *Dictyostelium* cells were able to survive with tau^{AP} expression. Since the constitutive expression of tau^{WT}, tau^{CR}, and tau^{17} rapidly induced cell death, we began using the pDM310 inducible vector system that would allow for the transcription of the tau gene to be reversibly turned on with the addition of doxycycline. My results show the successful construction of a tau^{WT} in pDM310 (+36pb TOPO) plasmid. This plasmid was transformed into *Dictyostelium* cells for expression of tau *in vivo*. Upon examination of tau^{WT} in pDM310 (+36pb TOPO) cell lysates generated after using several protocols for induction with doxycycline, we were unable to detect recombinant tau^{WT} protein. Further attempts to enable the expression of tau in *Dictyostelium* involved creating tau^{AP} and tau^{WT} in pDM310 constructs and transforming these into *Dictyostelium* cells. We are currently waiting for these tau in pDM310 cells to grow up to induce tau protein expression with doxycycline.

CHAPTER II

MATERIALS AND METHODS

Construction of pDXA vectors for constitutive expression of human tau and mutant tau in Dictyostelium cells

The experiments to insert the tau and mutant tau genes into the pDXA vector were conducted by Elizabeth Iocca, Amanda Erwin, and Sarah Hall from the Steinhilb lab and Kristi Miller from the Damer lab. The 1.2kb coding region of tau^{WT}, tau^{CR}, and tau^{AP}, and the 0.5kb coding region of tau^{17} were previously cloned into the pCDNA3.1 plasmid in the Steinhilb lab. Each of the tau cDNA clones were digested from the pCDNA3.1 plasmid and ligated into the *Dictyostelium* vector, pDXA, using KpnI and XhoI restriction enzyme sites. For restriction digests, a 20 µL mixture containing 1x New England Biolabs (NEB) Buffer 1, 1x BSA, 40 U KpnI, 40 U XhoI, 0.5-1µg of DNA, and water was made and incubated at 37°C for 1 hour. CIP (Calf-intestinal alkaline phosphatase 1:20) was added to the digested pDXA vector for an additional 1 hour at 37°C. The digested samples were mixed with loading dye (Promega) and run on a 1% agarose gel. The bands at 0.5 and 1.2kb indicating tau DNA and the band at 6.1kb indicating the pDXA vector were excised from the gel.

The tau^{WT}, tau^{AP}, tau^{CR}, tau^{17} and pDXA gel bands were purified using the QIAquick Gel Extraction Kit (QIAgen). DNA bands were excised from the gel and an amount of Buffer QG three times the mass of the gel band was added to the gel band in a microcentrifuge tube. The tubes were placed in a heat block at 50°C until all of the agarose had dissolved. Approximately 1 gel volume of isopropanol was added to the sample and then mixed. A QIAquick spin column was placed into a provided 2 mL collection tube and to bind the DNA, the sample was applied to the QIAquick spin column and centrifuged for 1 minute. The flow-through was discarded and the QIAquick column was placed back into the same tube. To wash the column, 0.75mL Qiagen

Buffer PE was added to the QIAquick column and centrifuged for 1 minute at 13,000 rpm in an eppendorf centrifuge 5418. The flow-through was again discarded and the column was centrifuged for another minute in order to remove residual ethanol from the PE buffer. The QIAquick column was placed into a 1.5 mL microcentrifuge tube and 30µl of autoclaved dH_20 was added to the center of the membrane and incubated for 1 minute. The column was centrifuged at 13,000 rpm in order to elute the DNA.

The gel purified DNA samples were analyzed using a NanoDrop 1000 Spectrophotometer to determine the relative difference in concentration between the samples and the ratio of vector to insert needed for ligations. Vector and tau insert DNA were mixed with 1x ligase buffer, 1.5 U T4 DNA ligase (Promega), and brought to a final volume of 10µL with autoclaved water. A 1 to 5 or 1 to 10 fold insert:vector molar ratio was estimated by the Nanodrop results. The ligation reaction was incubated at room temperature for 15 minutes.

Next, bacterial transformations were performed and 100-250 µl of NEB DH5α competent *E. coli* (High efficiency) cells were mixed with 10µl of the ligation reaction and then incubated on ice for twenty minutes. The tubes were heat shocked in a 42°C water bath for 2 minutes and then incubated on ice for 5 minutes. An addition of 950 µl of room temperature LB media (1% bactotryptone, 0.5% yeast extract, 1% NaCl) was dispensed to each tube and the tubes were then incubated at 37°C for 1 hour while shaking at 225-250 rpm. The transformation mixture was then spread on LB Amp plates (1% bactotryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 0.1mg/mL Amp) and allowed to dry. Plates were incubated at 37°C overnight and colonies were viewed the next day. Plates were placed at 4°C until ready to screen for tau in pDXA using colony polymerase chain reaction (PCR).

PCR primers were used that anneal to the 5' and 3' ends of the tau sequence. To amplify tau^{WT}, tau^{AP}, or tau^{CR}, a 20µl mixture was made with 0.25µM of the forward primer 5' FL tau NOT, 0.25µM of the reverse primer 3' FL tau Xho, 10µl Phusion (Thermo), a single bacterial colony, and autoclaved water. To amplify tau^{17}, a 20µl mixture was made with 0.25µM of the forward primer 5' tau 17KD FWD, 0.25µM of the reverse primer 3' 17KD Rev Xho, 10µl Phusion, a single bacterial colony, and autoclaved water. A negative control contained autoclaved water instead of bacteria. PCR conditions are listed in Table 3. Samples were stored at -20°C until analysis by gel electrophoresis.

Plasmid DNA from colonies from validated tau in pDXA clones was extracted using the QIAgen plasmid maxi kit. A small portion of a colony from a stock plate was transferred into a tube with 5 ml LB broth and 0.05 mg/mL Amp. These cultures were incubated at 37°C while shaking at 225-250 rpm. Approximately 8 hours later, 0.5 ml of of the culture was transferred to a 2000 ml flask containing 500 ml LB broth and 0.05 mg/mL of Amp. The culture was incubated overnight at 37°C while shaking at 225-250 rpm. Bacterial cells were harvested by centrifugation at 6000 x g in the Thermo Sorvall RC 6 Plus centrifuge for 15 minutes at 4°C. The supernatant was poured off and the bacterial pellet was resuspended in 10 mL of Qiagen Buffer P1. Next 10 mL of Qiagen Buffer P2 was added and mixed by inverting 4-6 times and then incubated at room temperature for 5 minutes. Chilled 10ml of Qiagen Buffer P3 was added and mixed by inverting 4-6 times and was then incubated on ice for 15 minutes. The sample was then centrifuged at 20,000 x g in the eppendorf centrifuge 5418 for 30 minutes at 4°C. The supernatant containing plasmid DNA was immediately removed and re-centrifuged at 20,000 x g for 15 minutes at 4°C and the supernatant containing plasmid DNA was removed immediately. A Qiagen-tip 500 was equilibrated by applying 10 ml of Qiagen Buffer QBT and the column was allowed to empty by

gravity flow. The supernatant containing plasmid DNA was then applied and allowed to enter the resin by gravity flow. The Qiagen-tip was washed twice with 30 ml of Qiagen Buffer QC. The DNA was eluted by adding 15 ml of Qiagen Buffer QF. The DNA was precipitated by adding 10.5 ml of room temperature isopropanol. The precipitate was collected by centrifugation at 15,000 x g for 30 minutes at 4°C. The supernatant was removed and the pellet washed with 5 ml of room temperature 70% EtOH and centrifuged at 15,000 x g for 10 minutes. Next, the supernatant was removed and the pellet was allowed to air dry for 5-10 minutes. The DNA was dissolved in 500µl autoclaved dH_20 and was stored in the -20°C freezer until ready for analysis by the NanoDrop 1000 Spectrophotometer to determine the concentration.

Cloning of tau and tau mutant genes into the TOPO plasmid

The 1.2kb coding region of tau^{WT}, tau^{CR}, and tau^{AP}, and the 0.5kb coding region of tau^{17} was amplified from the pCDNA3.1 vector using PCR. Primers were designed to add a BglII site and a FLAG tag at the 5' end and a SpeI site at the 3' end (Table 3). A 50µl reaction was set-up with 0.25µM of the forward primer, 0.25µM of the reverse primer, 25µl of Taq Go Mastermix (Promega), 1ng of the template pCDNA3.1 DNA, and autoclaved water. Negative controls were made by adding autoclaved water instead of DNA to one of the PCR reaction tubes. PCR conditions are listed in Table 3. PCR samples were analyzed by agarose gel electrophoresis and the rest was stored at -20°C until used for PCR purification.

PCR purification was performed using the Qiaquick PCR purification kit (Qiagen). One volume of the PCR sample was added to five volumes of Qiagen Buffer PB. A QIAquick spin column was placed into a provided 2 mL collection tube and the sample was applied to the QIAquick spin column and centrifuged for 30-60 seconds. The flow-through was discarded and the QIAquick column was placed back into the same tube. To wash the column, 0.75mL Qiagen

Buffer PE was added to the QIAquick column and centrifuged for 30-60 seconds at 13,000 rpm in an eppendorf centrifuge 5418. The flow-through was again discarded and the QIAquick column was placed back into the same tube. The column was centrifuged for an additional 1 minute at 13,000 rpm in order to dry the membrane. The QIAquick column was placed into a 1.5 ml microcentrifuge tube and 50 µl of autoclaved dH_20 was added to the center of the membrane and incubated for 1 minute. The column was centrifuged at 13,000 rpm in order to elute the DNA. An aliquot of the purified PCR sample was mixed with loading dye and then analyzed by agarose gel electrophoresis. The band at 1.2kb indicating tau^{AP}, tau^{CR}, or tau^{WT} DNA or 0.5 kb indicating tau^{17} was excised from the gel, purified using a QIAquick gel extraction kit, and then placed in the -20°C freezer until used for ligations.

The pCR2.1 TOPO vector (1µl/rxn) and 4µl of each gel-purified PCR samples were mixed with salt solution (1.2M NaCl, 0.06M $MgCl_2$) to reach a final volume of 6µL. The ligation reaction was incubated at room temperature for 5 minutes. For bacterial transformation, 3µl of the ligation reaction and 50 µl of NEB DH5α cells (High Efficiency) were mixed with and then incubated on ice for thirty minutes. The tubes were heat shocked in a 42°C heat block for 30 seconds and incubated on ice for 5 minutes. An addition of 950 µl of room temperature LB media was dispensed to each tube and the tubes were then incubated at 37°C for 1 hour while shaking at 225-250 rpm. During this incubation LB Amp plates were prepared for blue–white color screening by adding 40 µL of 2% X-gal on each plate. The transformation mixture was then spread on LB Amp plates and allowed to dry. Plates were incubated at 37°C overnight and colonies were viewed the next day. Plates were placed at 4°C until ready to perform overnight cultures and subsequent minipreps using a QIAprep spin miniprep kit (Qiagen).

Individual blue, white, and light blue colonies were picked into 5 ml of LB broth and 0.05 mg/mL Amp. The cultures were incubated overnight at 37°C while shaking at 225-250 rpm. The following day, 1.5 ml of the bacterial culture was placed into a microfuge tube, which was then centrifuged for 1 minute at 13000 rpm. The supernatant was removed and the pellet was resuspended in 250 µl of Buffer P1 and 250 µl of Buffer P2 was added and mixed by inverting 4 to 6 times. Next, 350 µl of Buffer N3 was added and mixed by inverting 4 to 6 times and the sample was then centrifuged at 13,000 rpm for 10 minutes. The supernatant was poured off and placed on a QIAprep spin column and centrifuged for 1 minute at 13,000 rpm in an eppendorf centrifuge 5418. To wash the column, 0.75mL Qiagen Buffer PE was added to the column and centrifuged for 1 minute.. The flow-through was discarded and the column was centrifuged for another minute in order to remove residual ethanol from the PE buffer. The QIAquick column was placed into a 1.5 mL microcentrifuge tube and 50µl of autoclaved dH_20 was added to the center of the membrane and incubated for 1 minute. The column was centrifuged at 13,000 rpm in order to elute the DNA. The eluted DNA was stored at -20°C until used for restriction enzyme digests.

Plasmids isolated from each colony were screened using BglII and SpeI double digests as well as EcoRI digests. A 10 µL mixture was made out of 1x NEB Buffer 2, 1x BSA, 10 U BglII, 10 U SpeI, 2 µl DNA, and water. Another 10 µL mixture was made out of 1x REact buffer 3 (Invitrogen), 1x BSA, 10 U EcoRI (Invitrogen), 2 µl miniprep DNA, and water. The samples were digested for 15 minutes at 37°C and then analyzed using agarose gel electrophoresis.

Preparation of tau and tau mutant genes in TOPO constructs for sequencing

About 1µg of DNA was mixed with dH_2O to bring to a volume of 6µL. Each sample was placed into a corresponding well in a 96 well plate; tau^{WT}, tau^{AP} , tau^{CR} and tau^{17} in TOPO were all sequenced using the M13 forward and M13 reverse primers provided by Michigan State University.

Cloning of tau and tau mutant genes into pDM310

The 1.2kb coding region of tau^{WT}, tau^{CR}, and tau^{AP}, and the 0.5kb coding region of tau^{17} were cloned into the SpeI site or the BglII and SpeI sites of the pDM310 vector. For the double digest, a mixture was made out of 1x Buffer 2, 1x BSA, 40 U BglII, and/or 40 U SpeI, 1µg tau in TOPO DNA and then brought to a volume of 40 µL with autoclaved water. The samples were digested for 15 minutes at 37°C and then heat inactivated for 20 minutes at 80°C on a heat block. The digested sample was mixed with loading dye and analyzed by agarose gel electrophoresis. The tau bands were excised and purified from the gel using a Qiagen purification kit. Gel purified samples were placed in the -20°C freezer until use in ligations.

To prepare the pDM310 vector for ligations, 0.5 µg of the vector was either digested with SpeI or a double digest was performed with BglII and SpeI as described above. The vector was digested for 15 minutes at 37°C and then heat inactivated for 20 minutes at 80°C on a heat block. Next, CIP (Calf-intestinal alkaline phosphatase 1:20) was added for 1 hour at 37°C. The sample was then purified using the QIquick PCR purification kit (Qiagen). The purified digested vector was placed in the -20°C freezer until use in ligations.

Small aliquots of purified linear pDM310 DNA as well as the gel-purified tau DNA were run on a gel to determine the relative brightness between the samples and the ratio of vector to insert needed for ligations. Vector and insert DNA were mixed with 1x ligase buffer, 1.5 U T4

DNA ligase (Promega), and brought to a final volume of 10µL with autoclaved water. A 1 to 2 or 1 to 3 or 1 to 1 fold insert:vector molar ratio was estimated via band intensity. The ligation reaction was incubated at room temperature for 15 minutes. Bacterial transformations were then performed followed by minipreps on individual bacterial colonies. After miniprep DNA was obtained, the tau in pDM310 candidates were screened using HindII and XhoI double digests as well as BglII and SpeI double digests. For HindII and XhoI double digests, a 20 µL mixture containing 1x NEB Buffer 2, 1x BSA, 40 U KpnI, 40 U XhoI, 0.5-1µg of DNA, and water was made and incubated at 37°C for 1 hour and then analyzed using agarose gel electrophoresis.

Transformation of plasmids into wild type Dictyostelium via electroporation

All tau plasmids were transformed into wild-type *Dictyostelium* cells via electroporation. *Dictyostelium* wild-type cells were washed off of plates and counted using a hemocytometer. The cells were pelleted by centrifugation in a Thermo Labofuge 400R tabletop centrifuge for 5 minutes at 2,000 rpm. The supernantant was removed and the cells were washed two times with 10ml of H50 buffer (20mM Hepes, 50mM KCL, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4). Once the cells were washed, the cells were brought to a concentration of $5x10^6$ cells/ml with H50 buffer. Next, 100 µl of cells and 10 µg of DNA were mixed in a sterile microcentrifuge tube and this cell/DNA mixture was subsequently placed into a 1mm chilled cuvette (VWR International). The cells were then shocked at 1400, 1450, and 1500 volts two times with 5 seconds in between each pulse using an eppendorf electroporator 2510. A control transformation was performed with wild-type *Dictyostelium* cells in the absence of DNA. After 5 minutes on ice, the cells were plated in 100x15 mm dishes containing 10ml of HL5 media. The following day G418 was added to select for plasmid-transformed cells.

Dictyostelium Growth Conditions

Cells were grown and maintained in tissue culture plates at 20°C. Approximately every seven days cells were diluted with Axenic HL5 medium (0.75% proteose peptone, 0.75% thiotone E peptone, 0.5% Oxoid yeast extract, 1% glucose, 2.5 mM Na2HPO4, and 8.8 mM KH2PO4, pH 6.5) with penicillin-streptomycin (Sigma) to facilitate growth. Plasmid transformed cells were grown and maintained with HL5 media containing 0.5 or 0.33 or 0.25µg/ml of G418 resistance to select for cells containing plasmid DNA.

Inducing the expression of tau with doxycycline

Tau^{WT} in pDM310 (+36bp TOPO) cells were harvested off plates and counted using a hemocytometer. Cells were diluted with HL5 media to make $4x10^6$ cells/ml. Cells were added to new plates and allowed time to attach to the bottom of the plate. The HL5 media was then removed from all of the plates and new HL5 media was added along with 25µl of 1mg/ml G418 to each plate. A new stock of doxycycline (Sigma) was made daily and 0.5, 10, or 20 µg/ml was added to cells every 24 hours. Protein extracts (described below) were taken at 0 to 4 days.

Preparation of protein extracts for Western blot analysis

Cells were harvested off plates and counted using a hemocytometer. Cells were then pelleted by centrifugation and were resuspended in 4x sample buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) to make 2×10^6 cells/lane. Samples were placed in a heat block and boiled at 95°C. All cell lysate samples were placed in the -20°C freezer until use in SDS-PAGE.

To prepare fly head controls, 3 flies were decapitated and the heads were placed in a microcentrifuge tube with 10 bashing beads and 30 µl Laemmli buffer (Sigma). The sample was

centrifuged for 45 seconds in an eppendorf centrifuge 5415D and then the sample was placed on ice for 45 seconds and the procedure repeated. Finally, the fly lysates were placed into a new microcentrifuge tube and boiled at 95°C for 5 minutes in a heat block. Fly lysates were placed in the -20°C freezer until use in SDS-PAGE.

SDS-PAGE and Western blot

Cell lysates and positive fly head controls were analyzed using a western blot. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and blocked with 10% RapidBlock solution (Amresco) for 5 minutes. The membrane was then probed with the monoclonal Tau-1 antibody (1:10,000, Millipore), which detects the presence of the tau protein, overnight while shaking at 4°C. Once the membrane was washed with 1x PBS-Tween to remove unbound primary antibody, the secondary horseradish peroxidase conjugated goat anti-mouse antibody (1:10,000, Pierce) was applied and the membrane was incubated for 1 hour at room temperature. Following further washes of the membrane with PBS-tween, enhanced chemiluminescence reagents (Thermo Scientific) were used to produce luminescence in proportion to the amount of protein. The Kodak Gel Logic 2200 was used for quantitation, image documentation, and analysis.

Preparation of cell lysate samples for PCR

Dictyostelium cells were harvested off plates and counted using a hemocytometer. Cells were brought to a concentration of 1 x 10^6 cells/ml with HL5 media. The cells were then pelleted by centrifugation and were resuspended in 25 µL of lysis buffer (50 mM KCl, 10 mM TRIS pH 8.3, 2.5 mM MgCl2, 0.45% NP40 and 0.45% Tween 20) containing Proteinase K (1 µl

of 20 µg/µl of Proteinase K for every 25 µl of lysis buffer). The sample was then incubated at 95°C for 5 minutes and subsequently stored in the -20°C freezer until PCR.

PCR to verify plasmid transformed cells

To determine if transformed *Dictyostelium* cells contained the plasmid DNA, PCR was performed to test for the presence of tau. PCR primers were used that anneal to the 5' and 3' ends of the tau sequence. A 50µl mixture was made with 0.25µM of the the forward primer 5' FLAG BglII , 0.25µM of the reverse primer 3' SpeI, 25µl Taq Go Mastermix, 1µl cell lysate, and autoclaved water. A positive control sample contained tau^{AP} in pDXA cell lysate. Two negative controls were used; one was wild-type *Dictyostelium* cells and the other contained autoclaved water instead of DNA. PCR conditions are listed in Table 3. Samples were stored at -20°C until analysis by gel electrophoresis.

Table 3. PCR conditions

	Primers	Reaction programs
Amplification of tau^{WT}, tau^{AP}, tau^{CR} for Colony PCR to screen tau in pDXA constructs	**5' FL tau NotI:** 5'-GATCGCGGCCGCATGGCTGAGCCCCGCCAGG-3' **3' FL tau Xho:** 5'-GATCCTCGAGTCACAAACCCTGCTTGGCC-3'	**STD3** **Denaturation:** 94°C-5 min **Annealing:** 35x 94°C- 15sec, 57°C- 30 sec, 68°C- 4 min **Extension:** 68°C- 5 min 4°C- hold
Amplification of tau^{17} for Colony PCR to screen tau in pDXA constructs	**5' tau 17KD FWD:** 5'-GATCGCGGCCGCATGAAAGCTGAAGAAGCAGGC-3' **3' tau 17KD REV Xho:** 5'GATCCTCGAGTCACAGATCCTCTTCTGAGATCAGTTTTTGTTCACGGACCACTGCCACCT-3'	**Tau17** **Denaturation:** 98°C- 5 min **Annealing:** 34x 98°C- 30 sec, 55°C- 1 min, 72°C- 45 sec **Extension:** 72°C- 10 min 4°C- hold
Amplification of tau^{WT}, tau^{AP}, tau^{CR} for cloning into pDM310	**5' FLAG BglII:** 5'-ATCGAGATCTATGGATTATAAAGATGACGATAAAGCTGAGCCCCGCCAGGAG-3' **3' SpeI:** 5'-ATCGACTAGTTCACAAACCCTGCTTGGC-3'	**FLMYTAUWT** **Denaturation:** 98°C- 30 sec **Annealing:** 34x 98°C- 10 sec, 65.4°C- 30 sec, 72°C- 2 min **Extension:** 72°C- 10 min
Amplification of tau^{17} for cloning into pDM310	**5' FLAG BglII tau^{17}:** 5'-AGATCTATGGATTATAAAGATGACGATAAAAAAGCTGAAGAAGCAGGC-3' **3' SpeI tau^{17}:** 5'-ATCGACTAGTTCAACGGACCACTGCCACCTT-3'	**MYCTAU17** **Denaturation:** 98°C- 30 sec **Annealing:** 34x 98°C- 10 sec, 67.3°C- 30 sec, 72°C- 1 min **Extension:** 72°C- 10 min 4°C- hold

CHAPTER III

RESULTS

Construction of pDXA plasmids to constitutively express human and mutant tau

In order to express human and mutant forms of tau in *Dictyostelium*, a pDXA plasmid was constructed containing the human tau or mutant tau genes under the control of the *Dictyostelium actin15* promoter. The *Dictyostelium actin15* promoter drives expression of tau in all cell types throughout development. The expression of *actin15* is present from 0 to 16 hours and then decreases at 20 hours (Knecht *et al.* 1986). The plasmid was also designed to contain a histidine tag at the 3' end of the gene to facilitate affinity purification. The 1.2kb coding region of tau^{WT}, tau^{AP}, and tau^{CR}, and the 0.5kb coding region of tau^{17} was digested from the pCDNA3.1 plasmid and cloned into the *Dictyostelium* vector, pDXA, using KpnI and XhoI restriction enzyme sites. The digested samples were run on a gel and the band at 1.2kb indicating the tau^{WT}, tau^{CR}, or tau^{AP} gene and the band at 0.5kb indicating tau^{17} and the band at 6.1kb indicating the pDXA vector were excised and gel purified. Ligations were performed using the gel purified DNA to covalently link the human and mutant tau DNA with the pDXA vector. Ligations were transformed into bacteria and colonies were screened using colony PCR. Positive clones were also verified by DNA sequence analysis (data not shown). Maxi-preps were performed on validated constructs in order to obtain sufficient quantities of DNA for *Dictyostelium* transformations.

Constitutive expression of tau constructs in Dictyostelium

Dictyostelium wild-type cells were transformed with tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} in pDXA plasmids through electroporation in which an electrical field causes an increase in cell membrane permeability allowing the plasmids to enter the cells (Knecht and Pang 1995). High

levels of G418 were used to select for cells with a greater number of plasmids taken up by the cell. Transformed cells were allowed to grow on plates until cells became confluent. Cells were then passed on to new larger plates. Cell viability was observed daily using microscopy. The constitutive expression of tau^{WT}, tau^{CR}, and tau^{17} was sufficiently toxic to induce cell death. *Dictyostelium* cells expressing tau^{WT} or tau^{17} survived approximately 2 weeks following the transformation while cells expressing tau^{CR} survived approximately 3 weeks. *Dictyostelium* cells were able to survive with tau^{AP} expression (Table 4).

Table 4. Cell viability analysis of *Dictyostelium* cells transformed with wild-type or mutant tau constructs.
After electroporation cells were plated in petri dishes containing 10mls of HL5 media and G418. Cells were observed daily via microscopy.

Constructs	Cell Viability (weeks)
tau^{WT}	2
tau^{17}	2
tau^{AP}	Survived
tau^{CR}	3

A Western blot was performed to verify tau^{AP} protein expression. Tau^{AP} protein expression was absent in *Dictyostelium* wild-type cells, which served as a negative control. Tau^{AP} protein of the expected size of about 60 kDa was detected in tau^{AP} *Dictyostelium* cells (Figure 3).

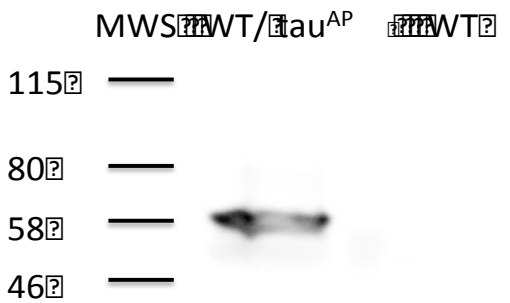


Figure 3. Western blot analysis of tau^{AP} protein expression in *Dictyostelium* wild-type cells.
Dictyostelium cells expressing tau^{AP} or wild-type cells were lysed in 4x sample buffer. $2x10^6$ cells were run on each lane of a 12% SDS-PAGE gel, transferred to PVDF membrane, and probed with a 1:10,000 dilution of tau1 antibody. A 1:10,000 dilution of goat anti-mouse antibody secondary antibody was used. The expected molecular weight sizes of a prestained protein marker are indicated in kDa at the left of the blot. Wild-type (WT) cells served as a negative control.

Due to the fact that the expression of tau^{WT}, tau^{CR}, and tau^{17} under a constitutive promoter rapidly induced cell death, we decided to use an inducible vector system in which transcription of the tau gene can be reversibly controlled with the addition of doxycycline. The goal was to clone tau^{AP}, tau^{WT}, tau^{17}, and tau^{CR} into the pDM310 vector using BglII and SpeI restriction enzymes. Initial cloning attempts were unsuccessful; therefore, we decided to instead clone the human and mutant forms of tau into the TOPO vector for the sole purpose of transfer into pDM310. Cloning the PCR fragments containing the BglII and SpeI restriction enzymes sites into the TOPO cloning vector allowed us to make sure that the ends of the fragments were being properly digested before ligating into the pDM310 vector.

Cloning human and mutant tau genes into the TOPO vector

The tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} genes were PCR amplified from the pCDNA 3.1 vector using a 5' BglII Forward primer to add on the BglII restriction enzyme site and a 3' SpeI Reverse primer to add on a SpeI restriction enzyme site (Table 3). A test gel was run to check for the presence of DNA and a distinct band was observed at the predicted size of 1.2 kb for tau^{AP}, tau^{WT},

and tau^{CR} and 0.5 kb for tau^{17} (data not shown). Each PCR reaction was first purified using the Qiagen PCR purification kit and an aliquot of each sample was run on a prep gel; the bands were then excised and purified. Another test gel was run to insure that the DNA was correctly purified. DNA was observed at the proper band size with tau^{AP}, tau^{WT}, and tau^{CR} at 1.2 kb and tau^{17} at 0.5 kb (data not shown).

A ligation was performed to covalently link the TOPO and tau^{AP}, tau^{WT}, tau^{17}, or tau^{CR} DNA, followed by bacterial transformations to introduce the plasmid into NEB DH5α cells (Figure 4). Blue-white color screening was used to determine which colonies contained the desired insert. Minipreps were performed on eighteen samples and then an EcoRI restriction enzyme digest was performed to screen tau in TOPO candidates. The digest samples were run on a gel and the various bands indicating the different sizes of DNA were analyzed. It was expected that bands would be observed at 3.9kb and 1.2kb for tau^{WT} and tau^{CR} in TOPO samples. Three bands were expected to be observed in tau^{AP} in TOPO samples; one band at 3.9kb indicating the TOPO vector, and bands at 0.8kb and 0.4kb indicating tau^{AP}, which contains an additional EcoRI site created by the point mutations from serine or threonine to alanine. It was expected that bands at 3.9kb and 0.5kb would be observed for tau^{17} in TOPO samples. DNA of the proper size was observed for tau^{AP}, tau^{WT}, tau^{CR} and tau^{17} (Figure 5). To further verify that the correct tau in TOPO constructs had been obtained, samples of tau in TOPO DNA were sent to Michigan State University for sequencing. The sequencing results revealed that we had created the correct tau^{WT}, tau^{AP}, tau^{CR} and tau^{17} in TOPO constructs.

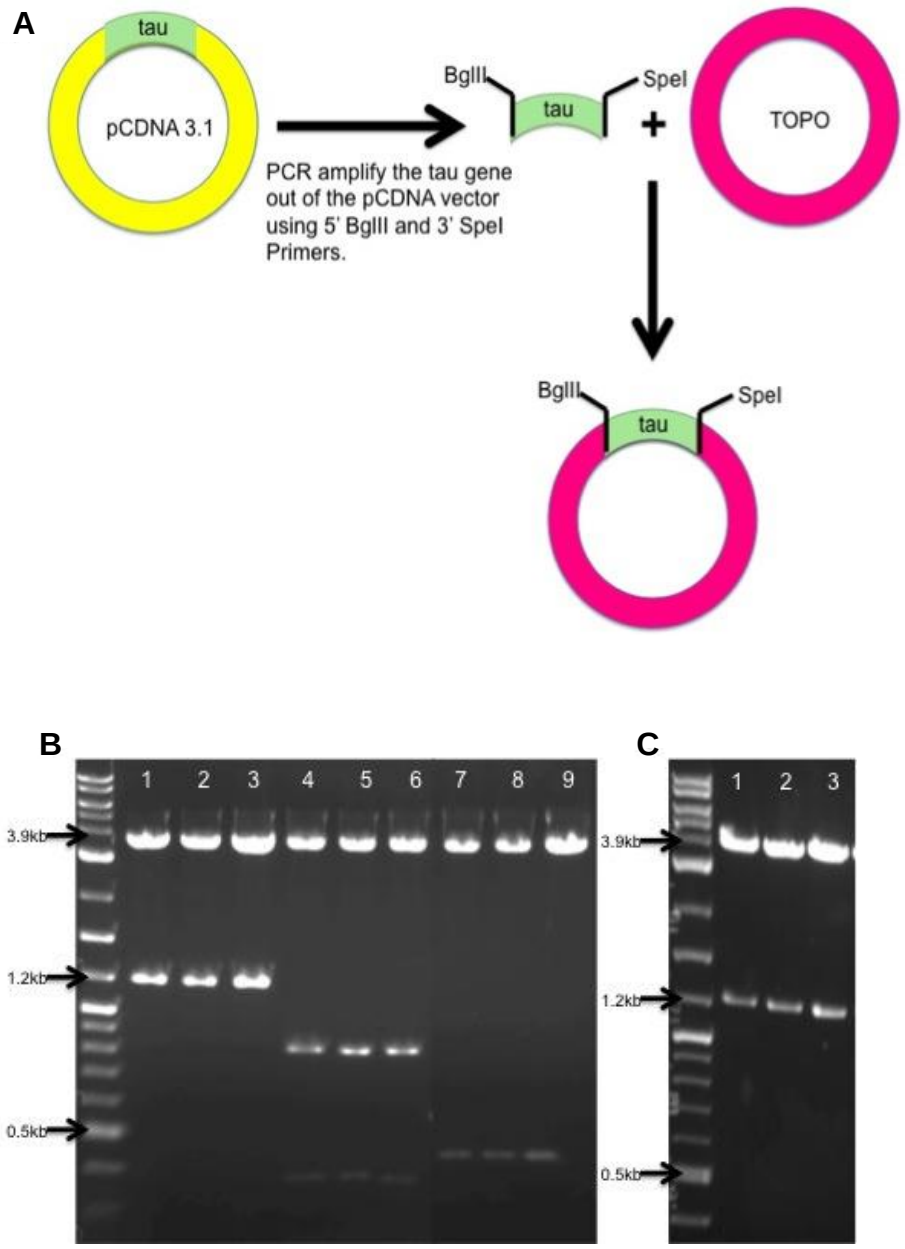


Figure 4. Construction and verification of tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} in TOPO.
A: Diagram of cloning human tau and mutant genes into the TOPO vector. B and C Test to screen tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} in TOPO candidates using miniprep DNA digested with EcoRI. B: Lanes 1-3 contain possible tau^{WT} in TOPO DNA, 4-6 contains possible tau^{AP} in TOPO DNA, and lanes 7-9 contain possible tau^{17} in TOPO DNA. C: Lanes 1-3 contain possible tau^{CR} in TOPO DNA.

The next logical step in the cloning process was to cut both the tau in TOPO DNA and pDM310 with BglII and SpeI restriction enzymes and perform ligations and transformations. This was unsuccessful, and was thought to be due to problems with using a double digest. A new plan of action was devised in which the human and mutant tau genes would be cloned into the pDM310 using only the SpeI enzyme. Cutting the tau in TOPO plasmids with just the SpeI enzyme would result in a fragment containing the tau gene as well as 36bp of the TOPO vector upstream of the start codon for the tau transcript (Figure 6).

Cloning the tau^{WT} gene into pDM310 using the SpeI enzyme

The tau^{WT} in TOPO sample was digested with SpeI to remove the tau^{WT} gene plus 36bp of TOPO DNA from the TOPO vector. The pDM310 vector was also digested using the SpeI enzyme in preparation for ligations. A prep gel was run with the digested DNA to check for the presence of DNA and a distinct band was observed at the predicted size of 1.2 kb for the tau^{WT} gene and 8.5kb for the pDM310 vector (data not shown). The bands were excised and gel purified. An aliquot of pDM310 and tau^{WT} (+36bp TOPO) purified gel DNA was then run on a test gel to check for the presence of DNA and also to compare the relative level of band brightness of insert to vector to determine the ratio of DNA needed for ligations. A ratio of 1:1, 1:2, and 1:3 vector to insert was used for ligations, which were performed to covalently link the tau^{WT} (+36bp TOPO) and pDM310 vector DNA. Bacterial transformations were performed to introduce the plasmid into the NEB DH5α cells. Bacterial colonies were then picked and minipreps were performed on eighteen samples. BglII and SpeI restriction enzyme digests were performed on the miniprep DNA to screen tau^{WT} in pDM310 (+36bp TOPO) candidates. After gel electrophoresis the correct band sizes of 1.2kb and 8.5kb were not observed (data not shown). At this point, after many failed cloning attempts, I posted a question to the Dictybase listserv to

determine if other researchers utilizing the pDM310 vector had encountered similar problems with cloning. I received several replies indicating that the pDM310 vector should not be gel purified, but should instead be purified using a QIAgen PCR purification kit after digestion (personal communication).

I followed the suggestions from the dictyBase listserv and purified a pDM310 sample using a QIAgen PCR purification kit after digestion with SpeI. Ligations were performed with the gel purified tau^{WT} (+36bp TOPO) DNA and the PCR purified pDM310 vector, followed by bacterial transformations to introduce the plasmid into the cells. Minipreps were performed on 18 bacterial samples and then a BglII or SpeI restriction enzyme digest was performed to screen tau^{WT} in pDM310 (+36bp TOPO) candidates. One sample after being cut with SpeI showed the correct size for pDM310 at 8.5kb and tau^{WT} (+36bp TOPO) at 1.2 kb, and after cut with BglII showed the correct size for linear tau^{WT} in pDM310 (+36bp TOPO) at 9.7kb (Figure 7). A maxiprep was performed on the validated tau^{WT} in pDM310 (+36bp TOPO) construct in order to obtain sufficient quantities of DNA for *Dictyostelium* transformations.

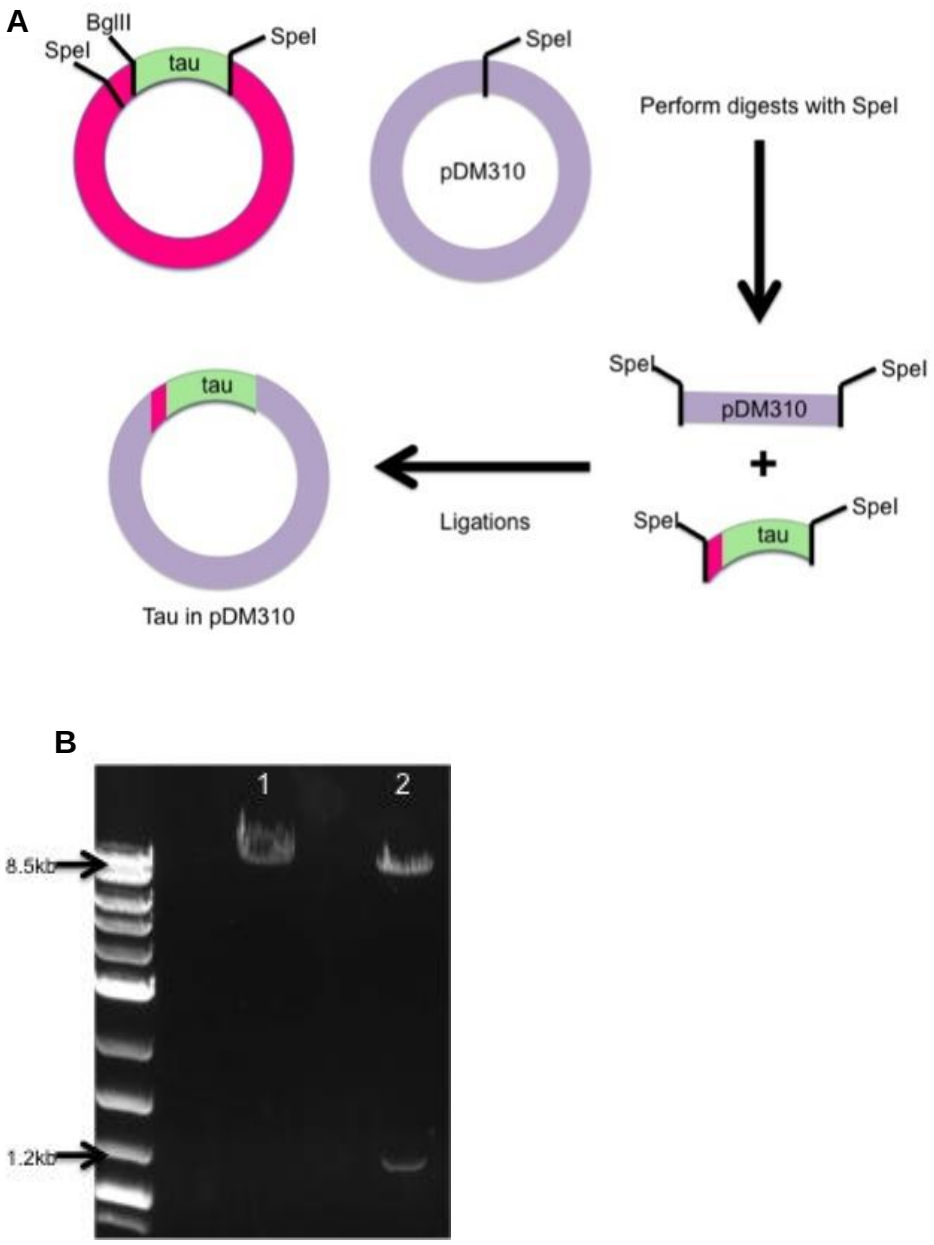


Figure 5. Construction and verification of tau^{WT} in pDM310 (+36bp TOPO).
A: Diagram of cloning human wild-type tau into the pDM310 vector using the SpeI restriction enzyme. B: Test to screen tau^{WT} in pDM310 (+36bp TOPO) candidates using miniprep DNA digested with BglII or SpeI restriction enzymes. Lane 1 contains tau^{WT} in pDM310 (+36bp TOPO) miniprep DNA digested with BglII, and lane 2 contains tau^{WT} in pDM310 (+36bp TOPO) miniprep DNA digested with SpeI.

Inducing the expression of tau^{WT} in Dictyostelium cells with doxycycline

Dictyostelium wild-type cells were transformed with the tau^{WT} in pDM310 (+36bp TOPO) plasmid through electroporation. High levels of G418 were used to select for cells with a greater number of plasmids taken up by the cell. Once a sufficient number of cells had grown up, doxycycline was added to the cells in order to induce the expression of the tau^{WT} gene. Concentrations of 0.5, 10, or 20 µg/ml doxycycline were added to cells and protein samples were taken at 0 to 4 days. A western blot was performed to verify the expression of tau^{WT}. A band at 60kDa was observed with tau^{WT} expressing *Drosophila* that served as a positive control. No tau^{WT} protein was detected with tau^{WT} in pDM310 (+36bp TOPO) transformed *Dictyostelium* cells (data not shown). These results led to the hypothesis that either the 36bp TOPO upstream of the start codon of the tau^{WT} construct was interfering with the induction process or the tau^{WT} in pDM310 (+36bp TOPO) plasmid was actually not present within the cells.

PCR with cell lysates to test for the presence of the tau^{WT} in pDM310 (+36bp TOPO) plasmid

To test for the presence of the tau^{WT} in pDM310 (+36bp TOPO) plasmid in the cells, PCR was performed with the 5' BglII Forward and 3' SpeI Reverse primers that anneal to the ends of the tau gene. Cell lysates were prepared from tau^{WT} in pDM310 (+36bp TOPO) cells maintained in HL5 media containing 0.25µg/ml or 0.33 µg/ml G418. Tau^{AP} in pDXA served as a positive control since the expression of tau^{AP} had been previously verified by western blot analysis. Wild-type *Dictyostelium* cells or a PCR reaction with water added instead of cell lysate served as a negative control. PCR reactions were performed with cell lysate samples and then samples were run on a gel. A band at 1.2kb was observed with both tau^{WT} in pDM310 (+36bp TOPO) samples as well as in the positive control. No bands were observed in the negative control lanes (Figure 8).

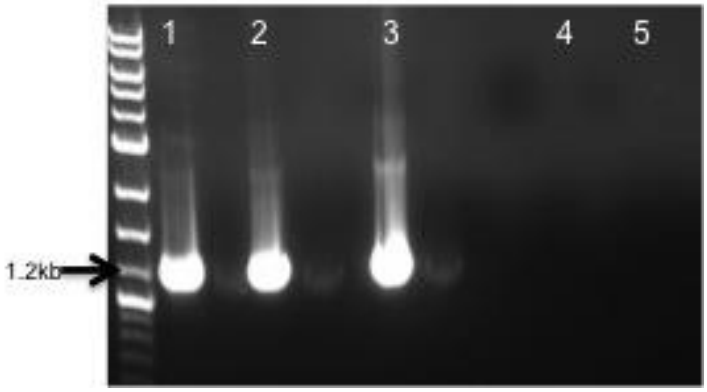


Figure 6. PCR analysis of wild-type *Dictyostelium* cells containing the tau^{WT} in pDM310 (+36bp TOPO) plasmid. Gel electrophoresis was used to show the results of PCR to test for the presence of the tau^{WT} in pDM310 (+36bp TOPO) plasmid. Lane 1 contains cell lysate of the tau^{WT} in pDM310 (+36bp TOPO) cells maintained in 0.25 µg/ml G418. Lane 2 contains cell lysate of the tau^{WT} in pDM310 (+36bp TOPO) cells maintained in 0.33 µg/ml G418. Lane 3 contains tau^{AP} in pDXA cell lysate, which served as a positive control. Lane 4 negative control WT cell lysate and lane 5 a negative PCR reaction containing water instead of cell lysate.

These results indicate the presence of the tau^{WT} in pDM310 (+36bp TOPO) plasmid within the transformed *Dictyostelium* cells. Therefore, it was hypothesized that the induction of tau^{WT} protein with doxycycline failed because the 36bp TOPO upstream of the start codon of the tau^{WT} construct was interfering with the induction process. To remove the 36bp TOPO as a possible problem we decided to instead clone tau into pDM310 using BglII and SpeI (Figure 9).

Cloning human and mutant tau genes into pDM310 using BglII and SpeI enzymes

The pDM310 vector was digested with BglII and SpeI enzymes and the sample was then purified using a QIAgen PCR purification kit. The purified pDM310 digest sample was run on a test gel to insure that the DNA was correctly digested and purified. A band of the predicted size at 8.5kb was observed (data not shown). The tau^{WT} and tau^{AP} in TOPO samples were digested with BglII and SpeI to remove the tau^{WT} or tau^{AP} gene from the TOPO vector. A prep gel was run with the digested DNA to check for the presence of tau^{WT} or tau^{AP} DNA and a distinct band was observed at the predicted size of 1.2kb. The band at 1.2kb was excised and gel purified. A ratio of 1:1, 1:2, and 1:3 vector to insert was used for ligations with the gel purified tau^{WT} or

tau^{AP} and the PCR purified pDM310 vector, followed by bacterial transformations to introduce the plasmid into the cells (Figure 9). Minipreps were performed on 18 bacterial samples and then a HindIII and XhoI double digest was performed to screen tau^{WT} or tau^{AP} in pDM310 candidates. Two samples (lane 1 and 4) showed the correct band sizes of 7.7kb, 1.39kb, and 0.5kb for tau^{WT} in pDM310. All three of the digested tau^{AP} in pDM310 samples showed the predicted band sizes at 7.7kb, 1.39kb, and 0.5kb (Figure 10).

The verified tau^{WT} and tau^{AP} in pDM310 plasmids were then transformed into XL10 Gold ultra competent cells in order to avoid problems with the possible rearrangement of the plasmid DNA. A maxiprep was performed to extract the plasmid DNA from the bacterial cells and obtain sufficient quantities of tau^{WT} and tau^{AP} in pDM310 DNA for *Dictyostelium* transformations. *Dictyostelium* wild-type cells were transformed with tau^{WT} and tau^{AP} in pDM310 plasmids through electroporation. High levels of G418 were added the following day to select for cells with a greater number of plasmids. We are currently waiting for these cells to grow up so that we can attempt to induce tau expression.

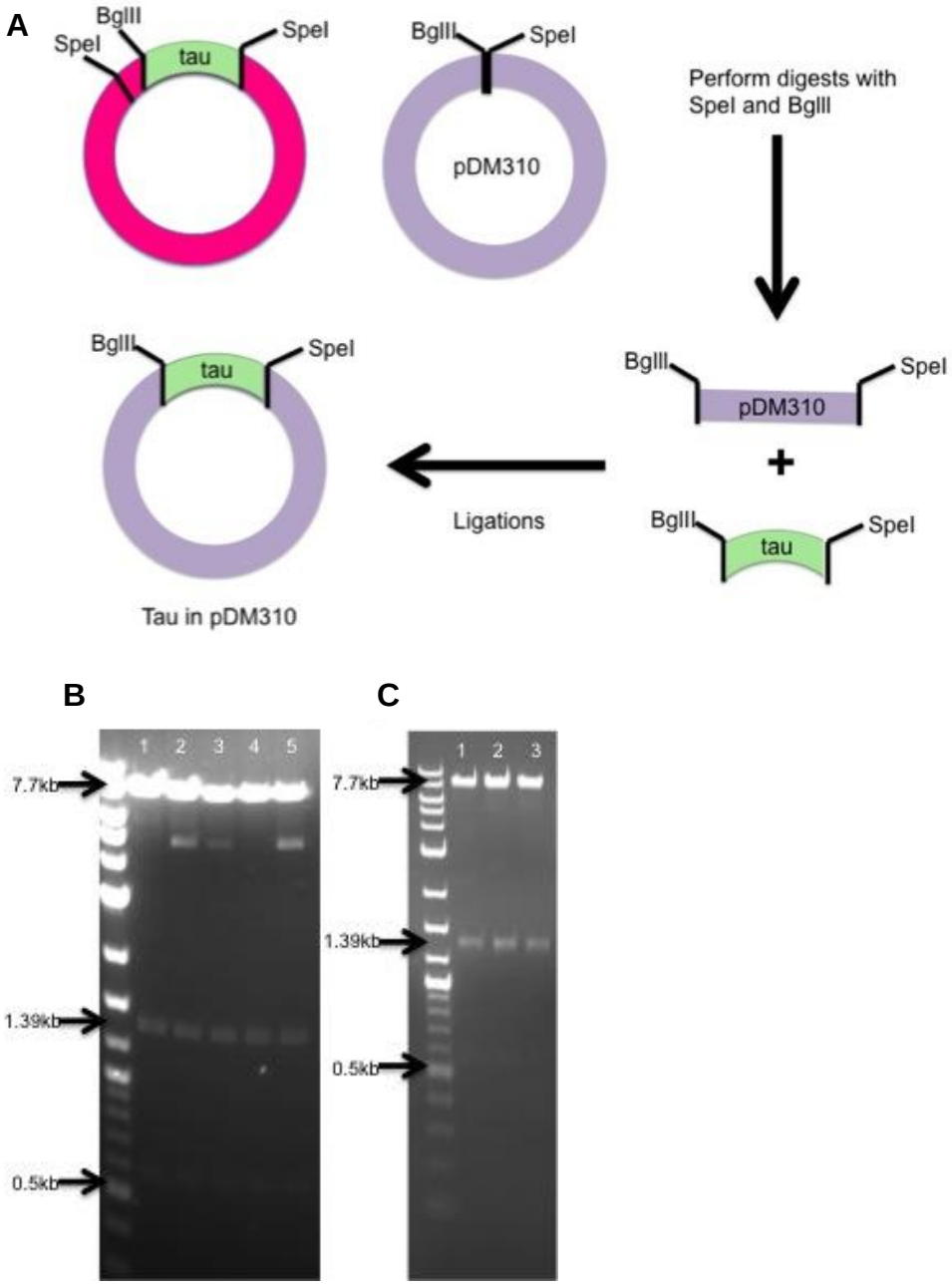


Figure 7. Construction and verification of tau^{WT} and tau^{AP} in pDM310.
A: Cloning of human tau and mutant genes into the pDM310 vector using SpeI and BglII restriction enzymes. B and C: Test to screen tau^{WT} and tau^{AP} in pDM310 candidates using miniprep DNA digested with HindIII and XhoI. B: Lanes 1-5 contain possible tau^{WT} in pDM310 DNAC: Lanes 1-3 contain possible tau^{AP} in pDM310 DNA.

CHAPTER IV

DISCUSSION

While studies have shown that tau is cleaved by calpain to produce a highly toxic 17kD fragment, it remains uncertain whether hyperphosphorylation plays a role in calpain cleavage (Park and Ferreira 2005). Research utilizing *Drosophila* has allowed the characterization of the toxic effects of human wild-type tau (tau^{WT}), phosphorylation-incompetent tau (tau^{AP}), calpain-resistant tau (tau^{CR}) and the 17kD tau fragment (tau^{17}) *in vivo* (Steinhilb *et al.* 2007; Reinecke *et al.* 2011; Whittmann *et al.* 2001). However, efforts to detect the 17kD fragment in recombinant tau protein samples *in vitro* remain unsuccessful (Steinhilb *et al.* 2007; Reinecke *et al.* 2011). A new expression system is needed to produce robust amounts of recombinant tau protein for biochemical analysis and allow the detection of the 17kD fragment. The aim of this study was to create constructs to express human wild-type and mutant forms of tau in *Dictyostelium*. It was hypothesized that using *Dictyostelium* would enable the high expression of recombinant tau protein and allow us to determine if phosphorylation regulates the cleavage of human tau and the production of the 17 kD fragment.

Our first aim was to create a plasmid that would allow the constitutive expression of human wild-type tau and mutant tau in *Dictyostelium*. tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} cDNAs were successfully cloned into a *Dictyostelium* pDXA vector and transformed into wild-type *Dictyostelium* cells. We expected that the more toxic the tau construct the greater defects would be observed on *Dictyostelium* cell viability. This prediction was based on the results of tauopathy studies utilizing *Drosophila* in which researchers expressed wild-type and mutant forms of human tau in the fly eye (Steinhilb *et al.* 2007; Reinecke *et al.* 2011; Whittmann *et al.* 2001). We predicted that wild-type *Dictyostelium* cells transformed with the tau^{17} plasmid would display the

most defects. The tau^{WT} plasmid would also be toxic to cells and disrupt *Dictyostelium* normal function. Cells transformed with tau^{AP} and tau^{CR} would exhibit fewer defects due to decreased tau phosphorylation and decreased cleavage of tau by calpain. Our results showed that the constitutive expression of tau^{WT}, tau^{CR}, and tau^{17} was sufficiently toxic to induce cell death. The constitutive expression of tau^{AP} was not toxic to *Dictyostelium* cells (Table 4). Western blot analysis confirmed that we had successfully created a line of *Dictyostelium* cells expressing the tau^{AP} protein indicated by the presence of a band at 60kDa (Figure 3). While there were never enough cells that survived to make protein extracts for a western to verify the expression of tau^{WT}, tau^{CR}, and tau^{17}, the fact that these cells lived for 2-3weeks before death suggests that they died as a result of a build-up of toxic tau protein. If cell viability had been negatively affected by the transformation process tau^{WT}, tau^{CR}, and tau^{17} cells would have been expected to die immediately after their transformation with the tau constructs. The addition of G418 would also have shown more immediate effects (2-3 days) if it was the cause of cell death.

The fact that cells were able to cope with the expression of phosphorylation-incompetent tau (tau^{AP}), but the expression of calpain-resistant tau (tau^{CR}) was sufficiently toxic to induce cell death may suggest that phosphorylation is a more important mediator of tau toxicity than cleavage by calpain. Further studies to analyze the *Dictyostelium* recombinant tau protein *in vivo* are required to support this. Additionally, it is possible that a protease other than calpain is cleaving the tau^{CR} protein in *Dictyostelium*, thereby inducing toxicity. *Dictyostelium* contains numerous serine proteases (tagB, tagC, tagA, tagD), which could in theory be cleaving tau^{CR} thereby making the protein toxic (Cabral et al. 2006; Anjard *et al.* 2011; Anjard and Loomis 2002).

It is also possible that the endogenous calpain in *Dictyostelium* recognizes and cleaves tau at sites other than K44 and R230 (Table 1). Little is known about the function of the calpain-like protein, Cpl, found in *Dictyostelium*. A study conducted by Huang (2003), first identified and purified the Cpl protein. Analysis of the structure of Cpl revealed homology to the mammalian calpain 10, which has been linked to type-2 diabetes (Lyn *et al.* 2002). Cpl was shown to exhibit minimal proteolytic activity by its ability to cleave small amounts of the substrate protein, casein. However, Cpl caseinolytic potential was significantly low in comparison to mammalian m-calpain or μ-calpain. This may indicate that Cpl has restricted substrate specificity or that is it does not act as a protease in *Dictyostelium* (Huang et al. 2003). Further studies need to be conducted to determine the exact functions of Cpl and whether Cpl proteolytic activity has an effect on tau toxicity in our tau expressing *Dictyostelium* cells.

Because the expression of tau under a constitutive promoter rapidly induced cell death, our next objective was to clone human wild-type and mutant tau genes into an inducible pDM310 vector that would allow for the transcription of the tau gene to be reversibly turned on with the addition of doxycycline. We were unable to directly clone wild-type and mutant tau genes into pDM310 using BglII and SpeI restriction sites, so we modified our plan and decided to clone tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} into the pCR 2.1 TOPO vector for the sole purpose of transfer into pDM310 (Figure 4). We chose to use TOPO cloning technology because it is highly efficient due to the fact that it utilizes DNA topoisomerase I, which allows for the ligation of PCR products directly into the TOPO vector without the need for a restriction enzyme digest of the PCR product. Human wild-type and mutant tau genes were ligated into the TOPO vector and then bacterial transformations were performed followed by minipreps to extract plasmid DNA. EcoRI digests were performed on tau in TOPO candidates and all samples showed the correct

size DNA fragments (Figure 5). Sequencing data also verified that we had created the correct tau in TOPO constructs (data not shown). The next logical step was to cut the tau genes from the TOPO vector and clone into pDM310 using BglII and SpeI. This was unsuccessful, and was thought to be due to problems with using a double digest. To overcome this problem, we decided to clone human wild-type and mutant tau genes into the pDM310 vector using only the SpeI enzyme. Cutting the tau in TOPO plasmids with the SpeI enzyme would result in a fragment containing the tau gene was well as 36bp of the TOPO vector upstream of the start codon of the tau transcript. This method of cloning was expected to result in a tau in pDM310 (+36bp TOPO) construct (Figure 6).

Attempts at making the tau in pDM310 (+36bp TOPO) construct were unsuccessful until we learned from the dictyBbase listserv that pDM310 should not be gel purified, but instead should be PCR purified before use in ligations. Gel purification requires a UV light to observe the presence of the DNA fragment before it is excised from the gel. PCR purification only requires the DNA to be run through a column. It is possible that the use of a UV light in gel purification is sufficient to mutate pDM310 DNA. We followed the suggestions from the dictyBase listserv and PCR purified the pDM310 sample after it had been digested with SpeI. Ligations with pDM310 and tau^{WT} were performed, followed by bacterial transformations and subsequent minipreps to extract plasmid DNA. A digest with SpeI or BglII was used to screen possible tau^{WT} in pDM310 (+36bp TOPO) samples. Upon gel electrophoresis it was determined that DNA of the proper size was observed at 9.7kb for the BglII digest and 8.5kb and 1.2kb for the SpeI digest (Figure 7). These results indicated that we had successfully created the tau^{WT} in pDM310 (+36bp TOPO) plasmid.

Wild-type *Dictyostelium* cells were transformed with the tau^{WT} in pDM310 (+36bp TOPO) plasmid via electroporation. High levels of G418 were used to select for plasmid-transformed cells. Once cells had gown up, doxycycline was added to the cells in order to induce the expression of tau^{WT} protein. Several concentrations of doxycycline were tested (0.5, 10, or 20 μg/ml) and protein samples were taken at 0 to 4 days after induction. A western blot was then performed to verify the expression of tau^{WT}. No tau^{WT} protein was detected (data not shown). We determined that there were three possibilities as to why we could not induce the expression of tau^{WT}: (1) The 36bp TOPO was interfering with the induction process (2) The tau^{WT} in pDM310 (+36bp TOPO) plasmid was not present within the cells or (3) there was a problem with the doxycycline induction process itself.

To determine if the tau^{WT} in pDM310 (+36bp TOPO) plasmid was present in the cells, PCR was performed using a forward primer that annealed to the 5' end of the tau^{WT} gene and a 3' reverse primer that annealed to the 3' end of the tau^{WT} gene (Table 3). Gel electrophoresis was used to visualize the amplified DNA from the PCR reactions. A band of the expected size of the tau gene at 1.2kb was observed with both tau^{WT} in pDM310 (+36bp TOPO) samples as well as in the positive control (Figure 8). These results indicated that the tau^{WT} in pDM310 (+36bp TOPO) plasmid was present within the transformed *Dictyostelium* cells. Therefore, it was hypothesized that either the induction of tau^{WT} protein failed because the 36bp TOPO upstream of the start codon of the tau^{WT} gene was interfering with the induction process or there may have been a problem with the doxycycline induction process itself. At this point, we were unable to obtain a positive control for the induction of protein expression in the pDM310 vector with doxycycline. Consequently, we were unable to test and determine if the induction process itself was a problem. To eliminate the 36bp TOPO as a possible problem, we decided to instead clone tau into

pDM310 using BglII and SpeI. While this method of cloning had failed previously, the new knowledge that the pDM310 vector should not be gel purified was expected to make this new cloning plan a success. The plasmid created from this method was expected to contain no TOPO DNA but only the tau gene in the pDM310 vector (Figure 9).

A pDM310 sample and tau^{AP} or tau^{WT} in TOPO were cut with BglII and SpeI enzymes. Ligations were then performed with tau^{AP} or tau^{WT} and pDM310 followed by bacterial transformations and subsequent minipreps (Figure 9). Tau^{AP} and tau^{WT} in pDM310 candidates were screened using a HindIII and XhoI double digest. Two tau^{WT} in pDM310 samples showed the expected size of 7.7kb, 1.39kb, and 0.5kb (Figure 10A, Lane 1 and 4). All of the screened tau^{AP} in pDM310 showed the correct size of 7.7kb, 1.39kb, and 0.5kb (Figure 10B). The verified tau^{AP} and tau^{WT} in pDM310 plasmids were then transformed into XL10 Gold bacterial cells, which are highly efficient and are designed to accept large plasmids and reduce the probability of rearrangements of plasmid DNA. A large-scale prep was used to extract the plasmid DNA from bacterial cells and then an electroporation was performed to introduce the tau^{AP} and tau^{WT} in pDM310 plasmids into wild-type *Dictyostelium* cells. G414 was added to select for plasmid-transformed cells and we are currently waiting for these cells to grow up enough so that we can attempt to induce tau protein expression.

Because we were unable to express human wild-type and mutant forms of tau in *Dictyostelium* we were unable to determine the validity of our hypothesis that using *Dictyostelium* would enable the high expression of recombinant tau protein and allow us to determine if phosphorylation regulates the cleavage of human tau and the production of the 17 kD fragment. However, this research is ongoing and the tau^{AP} and tau^{WT} in pDM310 plasmid-transformed cells will be used in future studies.

Future experiments with tau in pDM310 *Dictyostelium* cells will use a positive control to ensure that the doxycycline induction is working properly. We recently acquired a doxycycline-inducible extrachromosomal expression vector for expression of GST-Aardvark in *Dictyostelium* cells (Dickinson *et al.* 2011). The pGST-Aardvark vector will be transformed into wild-type *Dictyostelium* cells and then the conditions of our doxycycline induction protocol will be tested on these cells to express the GST tagged Aardvark protein. The presence of the GST tag added to the Aardvark protein allows for the easy detection of the expression of the Aardvark protein in cell lysates with the use of an anti-GST antibody in conjunction with a western blot.

Once we are able to express human wild-type and mutant tau in *Dictyostelium,* lambda phosphatase treatment will be performed on *Dictystelium* tau^{WT}, tau^{AP}, tau^{CR}, and tau^{17} cell lysates. Lambda phosphatase treatment will remove the phosphates from tau and allow us to determine the extent of phosphorylation of the recombinant tau synthesized in *Dictyostelium*. The total amount of phosphorylation on tau synthesized in *Drosophila* will be compared to *Dictyostelium* to ensure that the amount of phosphorylation is similar and the results obtained with these two model systems can be compared. Analyzing tau^{WT}, tau^{AP} and tau^{CR} post-phosphatase treatment for the presence or absence of the 17kDa fragment would allow us to determine the importance of phosphorylation on serine and threonine sites for proteolysis by calpain. If phosphorylation is regulating the cleavage of tau by calpain and the production of the 17kD fragment, it is expected that the 17kD fragment would be detected in tau^{WT} samples because this form of tau should be able to be phosphorylated and cleaved *in vitro*. It is expected that the 17kD fragment would be unable to be detected in tau^{AP} and tau^{CR} samples because these forms of tau contain mutations that inhibit tau phosphorylation or calpain cleavage (Steinhilb *et al.* 2007; Reinecke *et al.* 2011). If the 17kD fragment was detected in tau^{AP} samples this would

indicate that phosphorylation is not regulating the cleavage of tau by calpain. This would suggest that calpain cleavage of tau is possibly regulated by another post-translational modification such as glycosylation, nitration, or ubiquitination (Buee et al. 2000).

Future studies will also be conducted to characterize the effect of tau and mutant tau expression on *Dictyostelium* survivability, cell division, and development. Cell viability of plasmid-transformed cells will be assayed using a stain that only penetrates dead cells allowing the number of alive vs. dead cells to be counted within a population using a hemocytometer. Survival rates should be compared among the tau mutant cells as well as with wild-type cells. Additionally, plasmid transformed cells will be developed on both black nitrocellulose filters and water agar. These two media should be used because depending on environmental conditions development is altered. The effects of buffering, light, and moisture level all effect the development of *Dictyostelium* (Newell et al. 1969). It is expected that the more toxic the tau construct the greater the defect will be on *Dictyostelium* multicellular development.

Confocal microscopy and immunofluorescence will be used to observe the mechanisms of tau activity and changes in actin and tubulin. Specific antibodies will be utilized to view tau and tubulin within *Dictyostelium* cells while the stain rhodamine-phalloidin will be used to visualize actin. These assays will reveal the interactions between tau, actin, and microtubules within the cell and, more specifically, whether there is a co-localization of tau and microtubules, and tau and actin. We expect that tau expression in *Dictyostelium* will result in a disruption of the cytoskeletal network and it is assumed that with more toxic constructs there may be an increase in actin and tubulin defects. The changes to the cytoskeletal network in *Dictyostelium* will be viewed over time, as fixed samples will be made at various time points after the induction of tau expression.

While we were unable to express human wild-type and mutant tau in *Dictyostelium* in this study, research is ongoing and *Dictyostelium* may still prove to be an effective tauopathy model. We have transformed tau^{WT} and tau^{AP} in pDM310 plasmids into wild-type cells and will attempt to induce tau expression with doxycycline. We believe that *Dictyostelium* will enable the high expression of recombinant tau protein and allow us to determine if phosphorylation regulates the cleavage of human tau and the production of the 17 kD fragment. Using *Dictyostelium* as a tauopathy model may provide new insights into tau's role in neurodegeneration and provide hope for millions of patients with Alzheimer's disease.

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