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Understanding the effects of N-terminal acetylation on the oligomeric state of Tau

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Understanding the effects of N-terminal acetylation on the oligomeric state of Tau

An undergraduate project presented

By Miguel Martinez-Guzman

To the Biology Department

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ABSTRACT: Microtubule-associated protein Tau (MAPT) is a protein that is associated with dementias of the nervous system. These diseases are characterized by the abundance of Tau that is hyperphosphorylated and has aggregated into insoluble fragments. The post translational modification (PTM) mechanisms that regulate Tau are not working properly, and this protein has become pathogenic. In its functional state, Tau supports structural components used for transport inside of neurons. Tau's pathogenic state is affected by PTMs, but most research on Tau PTMs is focused on phosphorylation because Tau's microtubule affinity is regulated by phosphorylation. Tau is affected by PTMs such as acetylation, phosphorylation, and ubiquitination, N-terminal (Nt) acetylation is one such modification, the most common PTMs, with 80% of proteins humans modified this way. However, the effects that this modification has on each protein varies, so although it affects Tau it is not clear how. Bacteria cannot normally modify proteins post-translationally. However, with an *Escherichia coli* co-expression system that provides bacteria with the N-terminal acetyltransferases to acetylate the protein they create, it is possible to produce modified protein for study. Using fast protein liquid-chromatography to isolate the protein from bacteria, we can then use analytical techniques such as nuclear magnetic resonance (NMR), native polyacrylamide gel electrophoresis (PAGE), and Western blots will allow us to view the effects that Nt-actetylation has on the oligomerization rate and structure of Tau. Further elucidating the effects of N-terminal acetylation on Tau provides structural information about the use of the extreme N-terminus that is seen in microtubule binding proteins, and possibly grant a physiological marker for neurodegenerative diseases.

TABLE OF CONTENTS

Visualizing Protein

Future Directions

INTRODUCTION:

I. Dementia and Neurodegenerative Diseases

Dementia is one of the most common ailments that plagues our aging population, with over 55 million people worldwide suffering from it (WHO 2021). Characterized by a decline in mental health factors such as memory, learning, or social skills at a more rapid pace that is normally seen with age (Budson and Solomon 2011), it is a terrifying disease to face. With advancements in medicine, food production, housing, and hygiene, diseases like dementia whose greatest contributing risk factor is simply becoming older become more important to study to aid our aging population. One branch of dementia research is neurodegenerative diseases. Neurodegenerative diseases are a family of diseases that are characterized by gradual loss of function in neurons. Although the exact mechanism by which many of these neurodegenerative diseases operate through is unknown, enough information has been amassed concerning them to be able to identify either proteins of interest to study the disease, or in some cases even some genetic markers that mean an individual is predisposed to develop the disease later in life (Nikolac et al. 2019).

Some of the most common neurodegenerative diseases are Huntington's disease, Parkinson's disease, Alzheimer's disease, chronic traumatic encephalopathy, tauopathies, and multiple sclerosis. Despite this, neurodegenerative diseases display different symptoms and their prevalence among different populations varies, there are some commonalities within this family of diseases that allow for common research threads between them. All these diseases involve disruption of the protestasis network within the body, with each of them showing abnormal protein function in some way (Rubinsztein 2006). This gives a common thread through which they can all be researched through; information regarding abnormal protein homeostasis provides information for a variety of diseases.

Generally considered to be incurable because of the limited ability (or lack thereof) of neuronal cell recovery (Huebner and Strittmatter 2009), research in prevention of the diseases or being able to identify risk factors is relevant to stop any damage that these diseases may cause before it is significant. Being able to identify disruptions in the proteostasis of the body by observing changes that can occur with age would be a significant step in helping treatment and prevention of these diseases.

A. Chronic Traumatic Encephalopathy

Chronic Traumatic Encephalopathy(CTE) is a neurodegenerative disease that has been linked to repeated brain trauma (Ridler 2017). Its symptoms include cognitive impairment, changes in mood, and reduction in motor abilities. In the brain, this is shown off by a decrease of brain mass because of atrophy in the medial temporal lobe and frontal and medial cortices (Baugh et al. 2012). These symptoms do not show themselves immediately, and instead develop over an extended period. Originally being discovered in the early 20th century by studying boxers who were proclaimed "punch-drunk. Although this term was quickly phased out because of it trivializing the serious neurodegenerative disorder that was afflicting the boxers and replaced with *dementia pugilistica.* In the modern day, this disease continues to plague boxers, but it can be found in athletes and military veterans because of the tendencies for these individuals to suffer concussive trauma (Maroon et al. 2015).

Currently, there is no treatment or prevention for CTE. As it is linked to repeated brain trauma, the most effective treatment of it is currently to avoid successive trauma after initial injury, but there is no pharmaceutical or physical intervention to help with its symptoms. There are currently no reliable methods of being able to diagnose CTE before death, with the standard method of detection being observations made on a brain after autopsy. CTE shares symptoms with other neurodegenerative diseases, further compounding the difficulty in diagnosis (Askenes et al. 2017).

B. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease that results initially in memory loss, and eventually progresses to language issues, failure to regulate mood, and reduction in language capabilities

(Knopman et al. 2021). Diagnosis uses these symptoms along with patient family history, medical history to declare Alzheimer's disease (Klafki et al. 2006), Because of the similarities to other neurodegenerative diseases, a diagnosis by exclusion may be used as well, ruling out other diseases until AD is the only viable choice. Medical imaging techniques do exist that are able to recognize early onset Alzheimer's and to exclude the symptoms from other kinds of dementia, but these techniques are currently reserved for clinical trials. It is the most prevalent form of dementia in the world, making up 60-70% of all cases (Simon et al. 2018). AD is a mysterious disease, with its causes being mostly unknown, and as such ways to prevent the disease are unknown as well.

Although some research points to diet, exercise, pharmaceuticals, or education have been shown to help prevent AD, none of this data is conclusive and is too inconsistent to prescribe to patients that are in danger of developing the disease. Treatment for AD is in a similar state, with there being no cure for the disease, and most treatments seeking to alleviate symptoms instead of preventing further neurodegeneration or remission (McDade et al. 2021).

C. Huntington's Disease

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disease. HD begins with minor changes in mood and mental capabilities, and then develops into movement disorders resulting in need for long term care until the eventual death of the patient (NIH 2015). The symptoms appear at around 30 to 40 years old for normal HD, or earlier for Juvenile AD. After onset of symptoms, patients typically pass within 10 years.

The mutation that results in the disease is present on the *HTT* gene that codes for the Huntington protein (Dayalu and Albin 2015). In the wild type version of the protein, it contains a series of around 35 cytosine-adenine-guanine (CAG) repeats. However, there exists a CAG repeat domain expansion of the protein that results in a change of the conformation of the protein that increases its propensity to aggregate and leads to eventual formation of the disease (Walker 2007). Normally individuals with the disease are heterozygotes with only one copy of the mutant gene, but there have been reports of homozygous mutants that show worse and earlier symptoms (Squitieri et al. 2003). Although the function of the protein is not well known, it is assumed to have some function with axonal transport (Vitet et al. 2020). Although there are treatments to help with the symptoms of the disease, it is a terminal illness and after its appearance seldom can be done outside of palliative care to make the patient as comfortable as possible.

D. Parkinson's Disease

Parkinson's disease is a neurodegenerative disease that occurs because of the death of dopaminergic neurons. It attacks the central nervous system (CNS) (Kalia and Lang 2015). It is the second most ordinary form of dementia (Yao et al. 2013), after Alzheimer's disease. PD usually expresses itself initially as degradation of the body's motor systems. These symptoms include ataxia, stiffness, and trembling. As further degradation of the CNS occurs, more issues begin to arise, such as trouble eating, sleeping, talking and the development of depression (Kalia and Lang 2015).

Although PD is known to result because of the death of dopaminergic neurons, it is unclear through what mechanism the neurons die (NIH 2021). Although there is some controversy in the field as to what is a definite cause, a popular theory involves α-synuclein aggregation in the form of Lewy bodies (Schulz-Schaeffer 2010). Lewy bodies are clumps of protein within the brain that interfere with normal brain function and are considered the pathological biomarker for the disease.

Diagnosis of the disease is difficult, but the most reliable method of doing so has been to look at protein biomarkers such as dopamine metabolites and amino acids within the cerebrospinal fluid and blood serum. Medical imaging techniques also exist that can be used to help with the diagnosis, the deciding factor is blood tests and neurological evaluations (Armstrong and Okun 2020). Although we can identify the disease, the cause of it is still unknown. It is assumed that the disease is caused by a variety of environmental and familial factors. There is promising evidence to suggest that PD can exist as an inherited disease, with 15% of those afflicted having a relative with PD as well (Quadri et al. 2018).

There are currently no cures for PD because of the complicated onset of the disease, but there does exist pharmaceutical and surgical methods to reduce symptoms. In most cases surgical methods like Deep Brain Stimulation are avoided because of the risks associated with them, but in severe cases it is considered. Dopaminergic drugs are prescribed to assist with symptoms because the disease results from an irregularity of dopamine in the brain, but because dopamine cannot pass the blood-brain barrier and must be provided in a secondary manner. Drugs such as Levodopa, COMT inhibitors and dopamine agonists along with exercise are often prescribed as treatment (Armstrong Okun 2020).

II. Tau Structure and Function

Microtubule associated protein Tau is one of the proteins that is assumed to be involved in the proteinopathies (Alonso et al. 2001). In its functional state, the protein works to strengthen microtubules within the axons of the brain. Microtubules are important structural components of cells (Kapitein and Hoogenraad 2015) and help facilitate intracellular transport (Chaudhary et al. 2018). Tau strengthens microtubules by binding to polymerized microtubules through its microtubule binding repeat segments on its C-terminal domain. Upon doing so, the microtubules have their depolymerization rates slowed down, lowering the rate of degradation of the microtubules, and strengthening them (Cleveland 1977). Tau association with microtubules is dynamic, and it is regulated by post-translation modifications. Specifically, Tau can be phosphorylated on several of its residues, and this results in a lowering of its affinity to microtubules, allowing for a way for the cell to control Tau's activity (Alonso et al. 2018). However, hyperphosphorylation of the protein results in aggregation (Wang et al. 2013).Upon hyperphosphorylation, Tau is unable to bind with microtubules and instead forms Neurofibrillary Tangles (NFTs). These aggregates are part of the gold standard for the diagnosis of Alzheimer's disease. These insoluble aggregates form as a sort of "gunk" within the brain and does not allow for normal axonal function (Wang et al. 2013) Because Tau is also no longer strengthening the microtubules as it does in its native state, the microtubules in the brain begin to degrade as well (Cowan et al.2010) , resulting in the loss of a major structural component of axons. The effect of phosphorylation on the protein clearly indicates that post-translation modifications have a large effect on Tau.

Tau lacks a tertiary structure, being an intrinsically disordered protein. The single gene that encodes for Tau is located on chromosome 17 in humans and is named MAPT (Microtubule associated protein Tau). Alternative splicing results in six different isoforms of the protein, each distinguished by containing or lacking exons 2, 3, and 10 (Avila et al. 2016). Tau's functional domains are divided into sections based off the longest isoform of the protein, containing the N-terminal projection domain, the proline rich region, a microtubule binding domain, and a C-terminal domain. (Himmler et al. 1989). Because of this, the full-length protein is not required when conducting studies on the function of the protein or its activity in different conditions because it is assumed that the clinically relevant sections of the protein are involved with binding. However, as tauopathies are caused by abnormal oligomerization of Tau, research on the protein is focused on the C-terminal microtubule binding region of Tau that is involved with this abnormal oligomerization and this is also the region that is involved in microtubule interaction. This leads to other parts of the protein being understudied, while these ignored parts of the protein may serve some purpose.

A. Tau Function

Tau in humans is found primarily in neurons (Cleveland 1977). Inside of neurons, Tau plays a structural support role by assisting microtubules. Microtubules are tubulin polymers in the cytoskeleton of eukaryotic cells that provide rigidity (Ledbetter and Porter 1963). Tau interacts with microtubules by lowering the depolymerization rate, resulting in an increased growth rate and decreased degradation. The protein can exist in dendrites, but it is believed to be toxic (Hall et al. 2001). Dendritic cells are instead strengthened by the other main microtubule associated protein, MAP2 (Kalcheva et al. 1995)

Tau knockout mice do not display abnormal brain development and did not show an overtly deficient phenotype (Wang and Liu 2008) implying that Tau may not be an integral protein in microtubule stabilization, serving a redundant role along with other microtubule associated proteins. However, Tau KO

mice display lower muscle strength and psychological capability as they aged (Ikegami et al. 2000), implying that there is a role that other microtubule associated proteins cannot fill, or that Tau plays a regulatory role in maintaining brain function. Tau is regulated reversibly by the presence of phosphate groups, the addition or subtraction of these groups determining the microtubule binding affinity of Tau. In a healthy brain, phosphorylation is tightly regulated, and modification of Tau decreases the protein's affinity to microtubules (Mandelkow et al. 1995), with phosphorylation of S214 (Biernat et al. 1993), S262 (Scott et al. 1993), and S256 (Schneider et al. 1999) significantly lowering Tau's affinity to microtubules.

B. Membrane-less Organelle Formation

There exists a body of research that implies that before Tau forms its quaternary structures, there is a transition-state called liquid:liquid phase separation (LLPS). LLPS is the formation of membranelles organelles inside of cells. Like beads of oil that form in water, macromolecules can form spaces that are segregated from the rest of the cell. A protein phase separates when it is the most thermodynamically favorable state, and as such is dependent on protein/solution and protein/protein interactions (Franzmann and Alberti 2019). As such, acetylation may potentially affect the rate at which proteins phase separate by changing the total charge and protein/protein interactions.

It has been shown in *in vitro* studies that modifications to Tau that mimic disease-like versions of the protein increase the rate of phase separation (Kannan et al. 2020). In the same study, phase separated modified Tau has been seen to affect other soluble Tau protein in solution, increasing the rate of protein seeds that cause other proteins to become toxic, and the formation of pathogenic Tau that is seen before NFTs.

III. Post-Translational Modifications

Post-translational modification (PTMs) is one of the methods that the body employs to be able to regulate protein structure and therefore its function. These modifications change the chemistry of a protein reversibly or irreversibly and give the cell a method to toggle a protein "on" or "off," (Voet et al. 2006) or are a method employed in cell signaling to indicate something like oxidative stress (Wall et al. 2012). One common method of modification is the enzymatic modification of a functional group on a protein. After the protein has been produced by the ribosome, enzymes are able

to covalently modify proteins to modify their function. These modifications can occur on either the N or C terminus of a protein, or also on an amino acid residue (Voet et al. 2006). Typical modifications of this avenue are phosphorylation, acetylation, and ubiquitination.

A. Ubiquitination

Ubiquitination is a PTM that is a key event in the degradation of proteins by the proteasome, with the system that includes it being called the ubiquitin proteasome system (UPS). Misfolded, degraded, or otherwise nonfunctional protein are designated through this adenosine triphosphate (ATP) dependent method (Ciechanover 1998). Ubiquitination happens with three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3) (Pickart and Eddins 2004). These three enzymes working in tandem result in mono-ubiquitination, but normally Tau is polyubiquitinated before being recognized by the 26s proteasome and degraded (Babu et al. 2005). Polyubiquitination of Tau is catalyzed by CHIP with E2, and E3 provides specificity of the polyubiquitination (Galves et al. 2019). E3 creates an isopeptide bond between a lysine on Tau and the C-terminus of ubiquitin, so modifications of lysine residues in Tau may inhibit ubiquitination (Callis 2014).

However, instead of leading to the degradation of Tau, in PHFs found in AD brains Tau is highly ubiquitinated (Wang et al. 1991), implying that there is some connection with ubiquitination of Tau and its aggregation. Interestingly, most of the Tau that is found in AD brains is mono-ubiquitinated instead of polyubiquitinated (Cripps et al. 2006)

B. Phosphorylation

Phosphorylation is one of the most common and useful PTMs that can be applied to proteins. It is a reversible modification that activates or deactivates proteins (Cohen et al. 2002) Tau is a phosphoprotein; its function is modulated through the presence of phosphate groups, and it contains 85 potential serine, threonine, and tyrosine sites available for phosphorylation.

Tau is phosphorylated by many kinases, but they can be summarized into three main groups. These groups are the proline directed kinases (Hanger et al. 1992), non-proline directed kinases (Hanger et al. 2007), and tyrosine kinases (Williamson et al. 2002). With phosphorylation being a reversible modification, there is also a group of phosphatases that dephosphorylate Tau, such as protein phosphatase-1, -2A, and –5 (Liu et al. 2005)

Dysregulation of phosphorylation is seen in multiple neurodegenerative diseases. Normally, Tau is a disordered, highly soluble protein, but in various ND it exists in a highly ordered, insoluble, aggregated form. Phosphorylation is implied to be an upstream event of Tau aggregation, and hyperphosphorylated Tau aggregates spontaneously *in vitro* (Alonso et al. 2001), while dephosphorylation of soluble Tau from an AD brain restores native function of the protein (Iqbal 1998). Soluble Tau contains 10 phosphorylation sites when taken from a normal brain (Hanger et al. 2007), while Tauopathy brains show 16 phosphorylation sites (Wray 2008). However, it is currently unknown if it is overall phosphorylation or phosphorylation at specific sites that causes this toxicity. It has been observed that phosphorylation at specific residues is sufficient to trigger neurotoxicity in *Drosophila* (Steinhilb et al. 2007).

Another detriment of hyperphosphorylation is seen when Tau turnover rate is regulated by the degradation of the protein by the proteasome, and hyperphosphorylation has been seen to almost stop Tau degradation completely(Poppek et al. 2006). Increasing total protein level also increases the rate of aggregation within the cell to maintain equilibrium, so inhibition of degradation directly leads to more aggregates.

C. Acetylation

Acetylation is a modification that can occur post-translationally or co-translationally. Tau displays both N-terminal acetylation, and the ability to have some of its lysine residues acetylated (Min et al. 2010). Most of the lysine that have been shown to undergo acetylation are present in the MBD of Tau, and importantly acetylation of K280 is implied to be part of the transition to pathologic Tau by inhibiting microtubule binding and increasing the rate of PHF formation (Irwin et al. 2012) Unlike the acetylation of side chains, N-terminal acetylation occurs co-translationally (Dinh et al.2015). Most of the proteins inside of the eukaryotic proteome are N-terminally acetylated (Arnesen et al. 2009), which implies that there is some importance in the modification to the native function of a variety of proteins. Core metabolic processes display similar conservation across the eukaryotic spectrum (Peregom-Alvarez et al. 2009), so it may have vital importance to the function of proteins in the human body. However, the effects of N-terminal acetylation vary between proteins, which increases the difficulty of studying the modification.

Inside of the body, Tau is acetylated by the proteins p300 or the CBP acetyltransferase, and the acetyl group is sourced from acetyl-CoA (Min et al. 2010). Tau also displays some acetyltransferase activity, with some cysteine residues within the MBD (C291 and C322) showing the ability to acetylate Tau intra and inter molecularly. (Cohen et al. 2013)

There are two main consequences of acetylation that Tau is expected to exhibit. The first of these is the decrease of recognition by Tau by the UPS (Min et al. 2010). The lysine residues in Tau that have been observed to be regularly acetylated overlap with the residues that can be polyubiquitinated (Morris 2015), or the residues that work to designate Tau to be degraded. If the protein is not properly degraded and total Tau levels are increased, the amount of aggregates present will increase as well to maintain an equilibrium. Acetylation is also believed to inhibit native Tau-microtubule interactions. (Cohen 2011) Because acetylation works to neutralize the positive charge that is normally on the MBD of Tau, it impairs the ability of Tau to bind to the negatively charged surface of microtubules (Luo et al. 2014).

D. Effects of Aging on Protein Regulation

The human phenotype is plastic, as an individual ages, the functions of their body change as well. Going along with the main risk factor of neurodegenerative diseases being age, the function and number of various proteins and enzymes in our bodies decrease with age in accordance with decreased metabolism. Proteins lose the ability to be regulated reliably with age, and acetylation is one such process whose prevalence changes with age (Lu et al. 2011). Further studies into the effects of N-terminal acetylation can look at the relevance of this modification in the aging body.

IV. Proteinopathies and aggregation

Normally within a healthy brain, there are a wide variety of proteins that exist in their monomer form in a physiological and functional state. However, due to some cause these proteins undergo a conformational change and begin to create small oligomers and then eventually higher order structures within the brain. Proteinopathies are a family of diseases that are caused by irregular protein folding, and therefore irregular protein dynamics inside of the body. This irregularity disrupts the proteostasis network within the body. Within the body the proteostasis network is this tightly regulated system that allows the body to synthesize, distribute, and degrade proteins throughout the cytoplasm into locations where the protein is needed (Bayer 2015). Although Tau is a protein that does not have a tertiary structure, it is able to form these dangerous higher order structures. In some neurodegenerative diseases, when there is a stockpile of hyperphosphorylated Tau protein, it will spontaneously form insoluble aggregates known as paired helical filaments (PHFs) (Lee et al. 2001). Owing to its cysteine residues inside of the microtubule binding domain of the protein, Tau can spontaneously aggregate (Sahara et al. 2007). Although normally Tau can be disposed of by the proteosome and would be taken care of once it loses its function, aggregation lowers the ubiquitination of the protein and as such the aggregated protein cannot be disposed of and accumulates (Poppek et al. 2006).

A. Aggregation and Aggregates

Tau aggregates are called paired helical filaments because upon aggregation the proteins obtain a cross-β-sheet conformation with parallel, in-register β-sheets that run perpendicular to the long axes of the filament, resembling two protofilaments wound around each other. (Sawaya et al. 2007). The protein can form aggregates with alternative conformations (Maeda et al. 2007), but the PHFs are the most prominent aggregate seen, and resemble mature Tau that is recovered from AD brains (Berriman et al. 2003).

The aggregation of Tau *in vitro* is a reproducible event, so there is a secondary sequence characteristic that is driving Tau aggregation. Indeed, in a normal cell, Tau aggregation is driven by a hexapeptide motif (^{306}VQIVYK^{311}) contained in the third repeat region of the protein (Bergen et al. 2000). This region overlaps with the predicted highest β structure potential of Tau, and circular dichroism and Fourier transformation Infrared spectroscopy displays that in conditions of self-assembly that this region shows β structure character. (Bergen et al. 2000). It is believed that PHF formation is influenced by this sequence (Meraz-Ríos et al. 2010)

B. Toxicity of Aggregates

One of the common aspects of various neurodegenerative diseases is the toxicity of the aggregated proteins that are seen within the diseases. There is some controversy as to whether the NFTs are a contributing factor to neurodegenerative illness or simply an effect of them, with some research displaying soluble Tau as the culprit behind neurotoxicity, (Crimins et al. 2012) (Yoshiyama et al. 2007), but there is a gap in knowledge in the pathogenic mechanism of soluble Tau, which makes it difficult to study. However, although there are no Tau mutations that can be linked to an ND like AD, studies done on frontotemporal lobe degeneration have found >30 mutations in the MAPT gene that enhances aggregation and neurodegeneration (Swieten and Spillantini 2007). Interestingly, as well, in fetal human's Tau is hyperphosphorylated at rates like those seen in AD, but the protein does not show increased levels of aggregation (Kenessey and Yen 1993). Fetal Tau is comprised entirely of the third repeat region of the protein and has a lower rate of aggregation because of it. This implies that hyperphosphorylation and its

resulting loss of microtubule binding activity are not sufficient to cause a Tauopathy, and there are other factors that are involved in the illness's cascade.

The toxicity of Tau aggregates is observed in neurons where it is most abundant in the body. Upon emergence of disease, Tau can no longer properly bind to microtubules and cannot strengthen axons at an acceptable rate, leading to neuronal death (Wang et al. 2016). Native Tau function is vital for normal brain function.

Aggregated proteins have also been observed to act as a "seed" or template for other proteins, increasing the spontaneous aggregation rate once some protein has become misfolded, or if a misfolded protein is introduced to another environment. This is like prion diseases, where the misfolded protein can become a template for the other proteins in a cell (Walker 2013). Tau has shown this prion like ability, being able to propagate itself through adjacent cells (Dujardin and Hyman 2019). However, the mechanism by which Tau does so is unknown, and it does not propagate at a rate like other prions, so the protein is not considered a prion. Tau has also not proven itself to be infectious (Bloom 2014), so the protein is considered "prion like".

C. Oligomeric Species of Tau

Oligomers of a protein are protein conformations where there are a few repeating units of a protein put together. Although aggregates are looked at as the main culprit of cytotoxicity in cells, oligomers are another form of the protein that has been shown to be pathogenic. It has been seen that oligomeric species of Tau are an intermediate between the monomeric form of the protein and higher order species of Tau (Lasagna-Reeves et al. 2012). Higher order aggregates of the protein are insoluble and lose their native function, so the increase of an intermediate state would also increase the level of aggregates in cells. But even Prefibrillar Tau oligomers accumulating in the cell shows cognitive deficit that mirrors AD symptoms. (Mufson et al. 2014).

V. The Effects of Protein N-terminal Acetylation

This project aims to look at the changes N-terminal acetylation has on Tau structure and function. Although it has previously been displayed that N-terminal acetylation does influence the aggregation of this protein (Derisbourg et al. 2015), this is not a popular avenue of research when compared to phosphorylation of the protein, so there is a relative lack of information concerning this PTM. Analytical techniques such as western blotting, native PAGE, and NMR can be used to determine the effect on aggregation or oligomer formation that acetylation has.

A. Process of N-terminal acetylation

N-terminal acetylation involves the transfer of an acetyl group from acetyl coenzyme A to the alpha amino acid of a protein. Unlike most protein modifications, N-terminal acetylation is an irreversible modification. N-terminal acetylation occurs as the protein is being synthesized and is catalyzed by N-terminal acetyltransferases(NATs) associated with ribosomes (Gautschi et al. 2003). There exists a variety of NATs in eukaryotic systems, ranging from NatA-NatF, and each is composed of distinct subunits, granting them the ability to selectively acetylate proteins depending on their initial amino acid sequences(Polevoda et al. 2009).

B. Effect of N-terminal acetylation

Although acetylation is ubiquitous in humans with 80-90% of proteins that are produced in the body being acetylated (Drazic et al. 2016), it is not well understood what these changes do to the structure or the function of a protein. It has been hypothesized that N-terminal acetylation works to stabilize protein by blocking amino acids that could be marked for ubiquitination (Hershko et al. 1984), there has not been any proteins found that are degraded through N-terminal ubiquitination when lacking acetylation. Although an unacetylated N-terminus may lead to destabilization in a manner that is independent of the ubiquitin degradation pathway (Pena 2009). In humans, having a deficiency of NATs results in Ogden syndrome.

Children with Ogden syndrome are born with postnatal growth failure and pass within the first 1-2 years of life (Myklebust et al. 2015).

Evolution is an efficient process, so changes that are not necessary to the function of a protein are usually phased out, either to make space for a more efficient alteration or because the alteration is not necessary. Although higher eukaryotes express more NATs and as a result have a higher percentage of N-terminally acetylated protein, when looking at lower eukaryotes such as *Saccharomyces cerevisiae* exists a good deal of homology with the biological machinery of N-terminal acetylation (Polevoda 1999). Because acetylation is a highly conserved method of protein modification, it has some sort of relevant function in maintaining the function of proteins within the body .Additionally, Tau and other microtubule binding proteins contain an extreme N-terminus that is conserved in metazoans (Sündermann et al. 2016), but the role that this domain plays is unclear. There is some evidence that the N-terminus may fold over and be found around the hydrophobic core of the protein (Dulak et al. 2018). Folding in proteins is driven by the solubility of its amino acids in water (Zhou and Pang 2018). Changing the charge on the free amine group of an N-terminus to the neutral charge of an acetyl group has potential to change the way that Tau's extreme N-terminus is oriented in solution.

Affecting overall stability of the protein may have an effect on LLPS formation, as previously it has been displayed that unstable hyperphosphorylated species of Tau are both linked with disease and increase LLPS transition rate (Kanaan et al. 2020). Also, one aspect driving LLPS formation is electrostatic interactions between proteins (Boyko et al. 2019), so the change of charge could affect the rate at which LLPS and in turn oligomers of Tau form.

Acetylation modifies each protein that it affects uniquely. Generically, the addition of an acetyl group the free amine end of a protein changes the overall charge of a protein, affecting its electrostatic forces and make change how it interacts with itself. Although many proteins in the human body are seen to have this modification, the alteration that occurs when they are acetylated varies. Looking at other intrinsically disordered proteins for inspiration such as α- synuclein has its aggregation kinetics altered by N-terminal acetylation(Watson and Lee 2018). But this modification does not produce a noticeable generic effect, it must be studied specifically with a protein of interest to fully characterize its effects.

The hypothesis of this project is that N-terminal acetylation is a post-translational modification that changes Tau in a manner that affects its ability to oligomerize.

MATERIALS AND METHODS

Recombinant DNA

HB45: Nat A DNA in Pet15 Vector and Chloramphenicol Cassette

HB76: Full length Tau DNA in PET22 vectors with C-terminal His-tag and Ampicillin Cassette

Transformation of Plasmid into Competent Cells

Competent DE3 *E. coli* cells were thawed on ice for 10 minutes. 50 μl of competent cells were mixed with 1 μl of plasmid for 51 μl total volume. For double transformation use 2 μl of each plasmid because of low transformation rate, resulting in 54 μl total volume. Mixture was left on ice for 30 minutes before being heat shocked at 37°C for 90 seconds. After heat shock return mixture was returned to ice and left for 2 minutes. After 2 minutes 250 μl of LB was added, and mixture was incubated in shaker for 1 hour at 37°C. 75 μl of mixture was spread onto LB agar plates that have been brought up to 37°C. Plates contained appropriate antibiotic concentrations (2.9 mM ampicillin or 0.77 mM for chloramphenicol). Plates were inverted and then incubated overnight at 37°C or 48 hours at room temperature.

Large Scale Inductions

After successful bacterial transformation colonies were taken and put into 50 mL of LB with appropriate antibiotic (final concentrations 2.9 mM for ampicillin and 0.77 mM for chloramphenicol). After overnight incubation at 37°C, 5 mL of media was taken and placed into 4 1 L flasks of LB with appropriate antibiotics. Solution optical density (O.D) was measured at 600 nm until it reached between 0.2 and 0.4. Protein production in cultures were then induced with IPTG (0.8 mM final concentration) and left to incubate in shakers at 37°C for 24 hours.

Harvesting Large Scale Inductions

After induction, liquid cultures were moved to 250 mL centrifuge tubes and centrifuged at 10,000 RPM in a Sorvall™ centrifuge for 30 minutes. The resulting supernatant was discarded, and pellets were resuspended in 75 mL of water. In same centrifuge tubes, remaining liquid culture was added. After exhausting liquid culture from 1 L flasks, begin to combine resuspended pellets into 2 250 mL centrifuge tubes. This process was repeated until all culture was in 2 250 mL centrifuge tubes. Supernatant was again discarded, and cell pellets were stored at -20°C.

Protein Purification

Frozen pellets were thawed and resuspended inside of centrifuge tubes with lysis solution(50 mM Tris-HCL pH 9.0 150 mM NaCl 5mM DTT 1mM PMSF with one Thermo Scientific™ Pierce Protease Inhibitor Tablets). Pellets were sonicated on ice at 80% amplitude (Fisherbrand™ Model 120 Sonic Dismembrator) with 15 seconds of on time and 15 seconds of off time for a total time of 1 minute and 30 seconds. Pellets were subjected to two rounds of sonication with around 2 minutes in ice between sessions. After sonication pellets were moved to 90°C -100°C water bath for 15 minutes. After water bath, pellets were moved back to ice for 15 minutes. After resting on ice pellets were centrifuged for 45 minutes at 18,000 RPM at 4°C in Sorvall™ centrifuge. Supernatant were passed through a 20-micron filter into a 50 mL conical tube, and precipitate was discarded.

After this, protein purification with the ÄKTA™ start protein purification system using UNICORN software was began. The sample valve was placed into the 50 mL conical tube containing protein, and inlet valves were set up with one having buffer A (50 mM Tris-HCl 9.0 150 mM NaCl) and another with buffer B (50 mM Tris-HCl 150 mM NaCl 500 mM Imidazole). Column used was HisTrap™ 5 mL from Cytiva.

After setup, HIS-tag purification protocol was started. Sample volume was set lower than actual sample volume as to minimize the possibility of air entering FPLC. Fractions were automatically collected, and protein concentration was measured using UV absorbance. Protein solution was loaded onto column, and then wash was done with 100% buffer A for 10 column volumes. No fractions of interest are collected during this stage. Elutions were done with a linear increasing gradient of buffer B culminating in 100% buffer B over 5 column volumes. Fractions were divided into 5 mL each during elution. Fractions during elution stage of purification with high UV absorbance were denoted as samples containing Tau and collected, while other samples were discarded.

Protein concentration was then measured at 280 nm. If protein concentration was over 5 mg/mL it was diluted to 5 mg/mL and divided into 100 µl aliquots with 1 mM PMSF added. It was then frozen with liquid nitrogen, and stored at -80°C. If below 5 mg/mL, protein was concentrated in 5 kDa molecular weight cutoff Vivaspin® 20 Centrifugal Concentrator overnight at 15,000 RPM at 4°C overnight. On the following day protein was collected from concentrator and diluted to 5 mg/mL, and frozen with protocol outlined previously.

SDS-PAGE Visualization
Protein samples were run on 8% SDS-PAGE cast in house. 8 µl of PageRuler™ Prestained Protein Ladder, 10 to 180 kDa and 10 µl of sample in laemmli buffer was added to gel. Gel electrophoresis was run with Tris-Glycine SDS buffer at 100 volts for 10 minutes, and then 200 volts until dye front runs off gel. Gels were stained using standard Fairbanks procedure.

Native PAGE Visualization
Native PAGE gels were cast in house. 15 µl of Protein ladder was ran in Native PAGE gels to visualize progress of gel. 15 µl ofProtein samples were ran in native running buffer (47% sucrose, 3 nM Tris 3 nM PIPES 1 nM EDTA). Empty lanes received 15 µl of native running buffer. Gel was run in native running buffer (30 mM Tris 30 mM PIPES 10 mM EDTA), for 3 hours at 4°C at 100 volts, or under the same conditions at 30 mAmperes. Gels were stained with normal Fairbanks procedure.

Western Blot
Trans-Blot® Turbo™ mini size ion reservoir stacks were equilibrated in SDS transfer buffer (0.025 M Tris-OAc pH 8.8 0.92 M Glycine 0.35 mM SDS) for 2-3 minutes. Immun-Blot® Low Fluorescence PVDF Membrane was placed in methanol until translucent and then transferred to tray with SDS transfer buffer and allowed to equilibrate for 2-3 minutes. Sandwich was assembled with gel from SDS page in cassette and ran on Trans-Blot® Turbo™ included protocol for mini gels. After transfer protein was blocked with a 1x solution Blocker™ BSA (10X) in TBS at 4°C overnight.

After overnight blocking, membrane was rinsed for 5 minutes with 0.1% TSBT (20 mM Tris 150 mM NaCl 0.1% Tween 20). Membrane was then washed with a 1:8,000 dilution of Tau Ab-3 rabbit polyclonal antibody in 1x TBS (50 M Tris 150 mM NaCl) for 45 minutes. After primary antibody wash, membrane was washed 3 times with 0.1% TBST for 10 minutes. Then, the membrane was tagged with either a 1:5,000 dilution of Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP for chemiluminescence or a 1:500 dilution of Goat Anti-Rabbit IgG H&L (DyLight® 650) for fluorescent studies. Both antibodies were diluted in 1x TBS.

After secondary antibody tagging membrane was washed 3 times for 5 minutes each with 0.3% TSBT (20 mM Tris 150 mM NaCl 0.3% Tween 20), and then for another 3 times for 5 minutes with 0.1% TSBT. For chemiluminescence appropriate amounts of ECL reagent was added to cover membrane. Both membranes were visualized using Azure 400 imager's included settings.

RESULTS

Establishing Double Plasmid Transformation Efficiency and Protein Expression

My first goal was to establish the effectiveness of transforming two plasmids with two different selection markers in E. coli. To accomplish this, *E. coli* colonies were selected on LB plates with both ampicillin and chloramphenicol. Although growth on the plate was less than what would normally be expected from a culture (Fig. 1A), any growth on the plate indicates that the double transformation was a success, and that it can be moved onto a larger scale system.

Following the successful growth on agar, bacterial colonies from these plates were taken and the protein produced and purified from them was visualized on SDS-PAGE. The additional acetyl group on the modified protein Tau will alter its migration when resolved by SDS-PAGE when compared to the unacetylated protein, which is normally seen at around 75 kDa. This change in migration is observed by SDS-PAGE (Fig 1B) where the major protein band from a purification of acetylated Tau runs higher than the unmodified protein.

Purification of Tau/Acetylated-Tau from *E. coli*.

After confirming that the transformation and Tau expression was successful, I then scaled up the expression cultures to produce larger amounts of pure Tau. From a culture that was taken from a successful transformation, samples were taken before and after induction with IPTG. A large band at 75 kDa appears after Tau induction (Fig 2A), indicating successful start of protein production. Producing protein with a C-terminal poly-histidine motif enables purification from lysed cell culture through nickel affinity chromatography. Each step (Fig. 2A) taken in the purification looks to further isolate Tau from unwanted products of bacterial production, such as cell debris or miscellaneous proteins. The product of this isolation is mostly pure Tau protein (Fig. 2B and 2C).

One benefit of working with an intrinsically disordered protein is the ability to boil samples. Like boiling egg whites, proteins that are exposed to high-temperature environments unfold and lose their solubility, crashing out of solution. However, Tau's status as an intrinsically disordered protein means that we can boil it and it remains soluble in solution. Many proteins that *E. coli* produce are precipitated during this boiling process, and you can see that after boiling the major band that corresponds to Tau at 75 kDa remains with other disappearing. Following boiling, I used FPLC and nickel affinity chromatography to isolate Tau from solution. I measured the protein content of the flowthrough by measuring UV absorbance of sample (Fig. 3A and 3B), leading me to collect fractions from elution steps that have high absorption values, such as 7-11 (Fig. 3A) for full-length Tau and fraction 9 (Fig. 3B) for acetylated Tau. Total protein content correlates to the integral of the fractions that are being collected, so although both purifications loaded similar amounts of sample onto column, the full-length Tau has an area of 3,733 mAU while acetylated Tau has an integral of 869 mAu. The full-length Tau sample provided around 4 times as much absorbance. This is believed to be a limitation of the double-transformation system, producing significantly less protein when compared to a single transformation.

Determining optimal storage conditions for purified Tau

Although we can produce large quantities of protein with every successful induction, my next goal was to establish proper storage conditions for Tau. Acetylated Tau in particular produces more dilute fractions than full-length Tau because of the reduced total protein production. My goal was to concentrate Tau to 5 mg/mL, and protein fractions that did not meet this were concentrated via spin concentrator tubes. Increase of total protein in solution was seen after overnight concentration. (Fig. 4A)

To store protein for extended periods of time, special measures must be taken, but they each have their trade-offs. Concentrated full-length Tau samples from a purification were diluted and treated to a variety of conditions over 48 hours to view degradation of protein (Fig. 4B). Tau that was frozen in liquid

nitrogen and then stored at -80°C showed the least amount of degradation. Although the sample from protein precipitate shows low amounts of degradation as well, the process to precipitate proteins is more time-consuming, and results in some protein being lost. Also, although storing with glycerol does also prevent a good deal of degradation, introducing glycerol to assays can cause issues, so working with minimal or no amounts is ideal. Because of its ease of use and effectiveness, the liquid nitrogen method was determined to be optimal for long-term storage.

Visualizing Tau Oligomers Found in Solution

While SDS-PAGE resolves denatured proteins, this means that higher-order structures or electrostatic interactions between proteins in sample cannot be visualized. My goal is to determine changes to Tau's oligomeric state and as such, I utilized native gel electrophoresis to observe samples in their native states. Protein was successfully resolved by native PAGE (Fig.5A) and I experimentally determined optimal conditions to maximize resolution. Varying concentrations of Tau were run to determine the best concentration of protein to use (Fig. 5B), and 2.5 mg/mL provided sufficient resolution. I next wanted to optimize the resolution of the separated Tau oligomers and my first task was to vary the concentration of acrylamide in the native gel. While lower percentages allow larger protein complexes into the gel, there is significant loss of resolution. I experimentally determined with Tau that 10% acrylamide was sufficient for the native PAGE (Fig. 5C). Another variable that affects resolution is the electrical current running through the gel. I initially ran gels under constant, however this did not sufficiently resolve oligomeric complexes and resulted in smears. I next set the conditions to constant amperage, which would result in a consistant current as the proteins migrant within the gel. Running the gel at a constant amperage with all other conditions equal provided a gel with increased resolution. With a 10% gel and a constant amperage I was able to resolve monomers and dimers of Tau (Fig. 5D)Tau. While these are preliminary pilot experiments, being able to separate proteins based on their molecular weight allows me to view dimers, trimers, and other higher order structures for to quantify in future experiments.
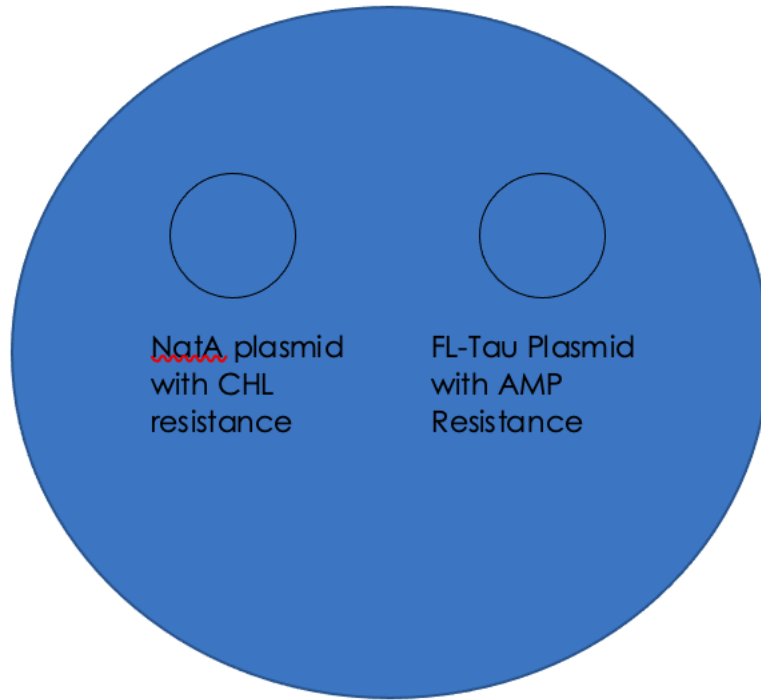
Visualizing Protein by Western blot

Visualizing proteins by native gel electrophoresis requires a significantly higher concentration of protein and we may not be able to visualize oligomers that are at low abundance. To address this, we utilized Western blotting using a conformation agnostic anti-Tau antibody to visualize all species of Tau found in solution. Full-length Tau that was purified and resolved by SDS-PAGE was transferred to a low fluorescence PVDF membrane. The membrane was tagged with a primary antibody of rabbit anti-Tau and followed with a goat anti-rabbit antibody conjugated with HRP for chemiluminescence. After tagging of secondary antibody the membrane was covered in ECL reagent. For fluorescence, instead of the goat anti-rabbit conjugated with HRP, the membrane was instead tagged with a goat anti-rabbit antibody that was tagged with a fluorescent marker.

Tau was visualized successfully utilizing secondary antibodies by both chemiluminescent (Fig. 6A) and fluorescent methods (Fig. 6B). Although the SDS-PAGE that the Western blots are preliminary, I was still able to visualize Tau using two separate methods.

A

NatA plasmid
with CHL
resistance

FL-Tau Plasmid
with AMP
Resistance

B

150 kDA

120 kDA

95 kDA

75 kDA

65 kDA

43 kDA

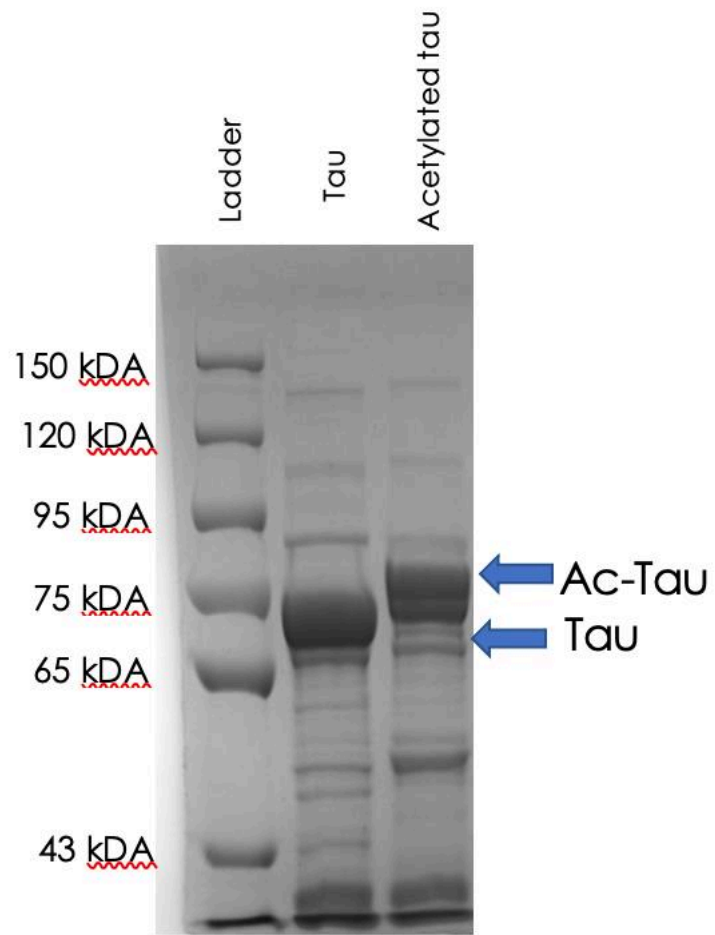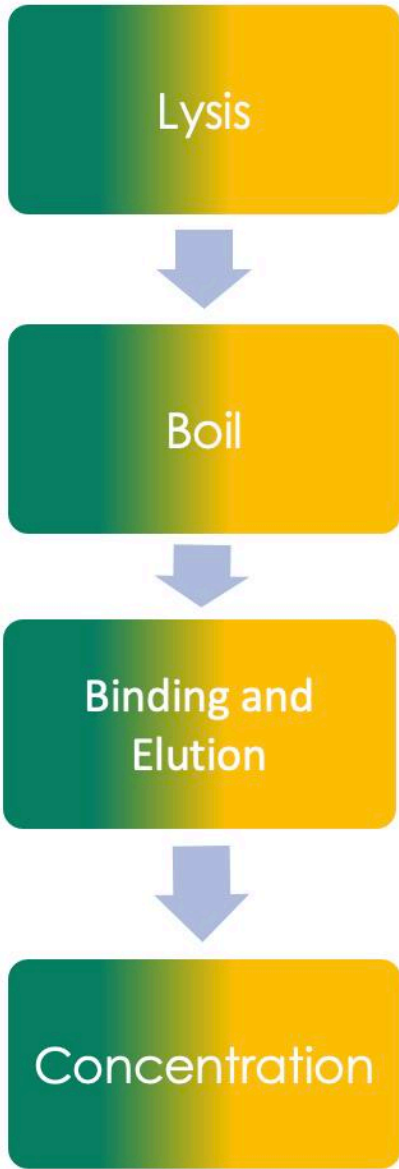Ladder   Tau   Acetylated tau

Ac-Tau

Tau
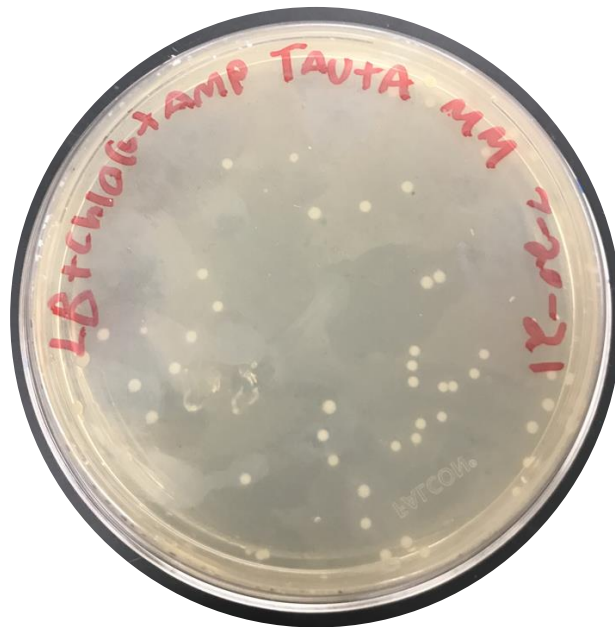
Figure 1: Protein expression gel of Full-Length Tau (FLT) and Acetylated Tau (AT) A. Schematic Diagram of Double Transformation with Agar Plate Confirming Transformation Two plasmids can be inserted into *Escherichia coli* in order to modify protein in a bacterial expression system. Agar plate contains antibiotic selecting for each plasmid. **B. Full Length-Tau and acetylated Tau Expression Gel** 8% SDS PAGE ran to visualize the migration of N-terminally modified Tau compared to unmodified. Stained with Coomassie blue.

A

Lysis

↓

Boil

↓

Binding and Elution

↓

Concentration

B

Ladder | Cell Pellet (Uninduced) | Cell Pellet (Induced) | Cell Lysate | Boiled Sample | Flow Through | Wash Solution | Elution

150 kDA
120 kDA
95 kDA
75 kDA
65 kDA
43 kDA

← Tau band

C

Ladder | Cell Pellet (Induced) | Cell Lysate | Cell Lysate | Cell Lysate | Flow Through | Wash | Elution

150 kDA
120 kDA
95 kDA
75 kDA
65 kDA
43 kDA

← Tau band
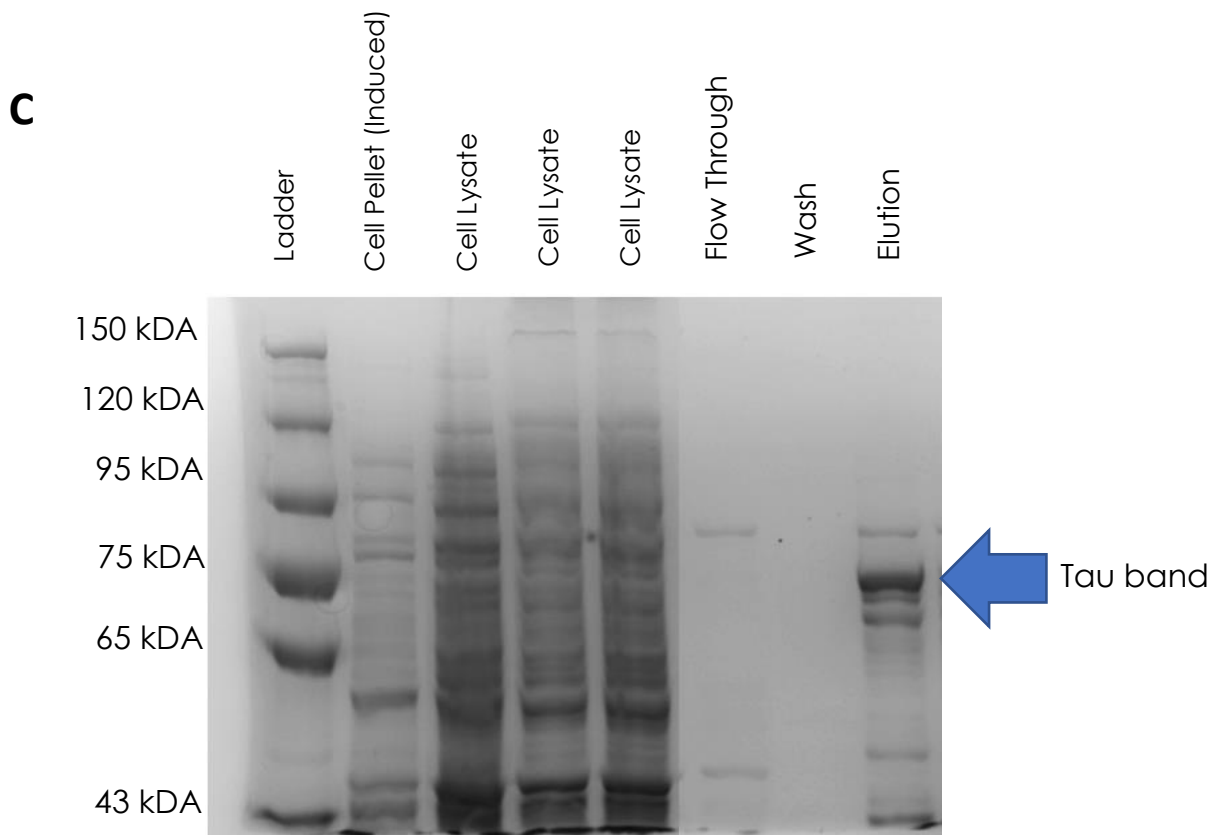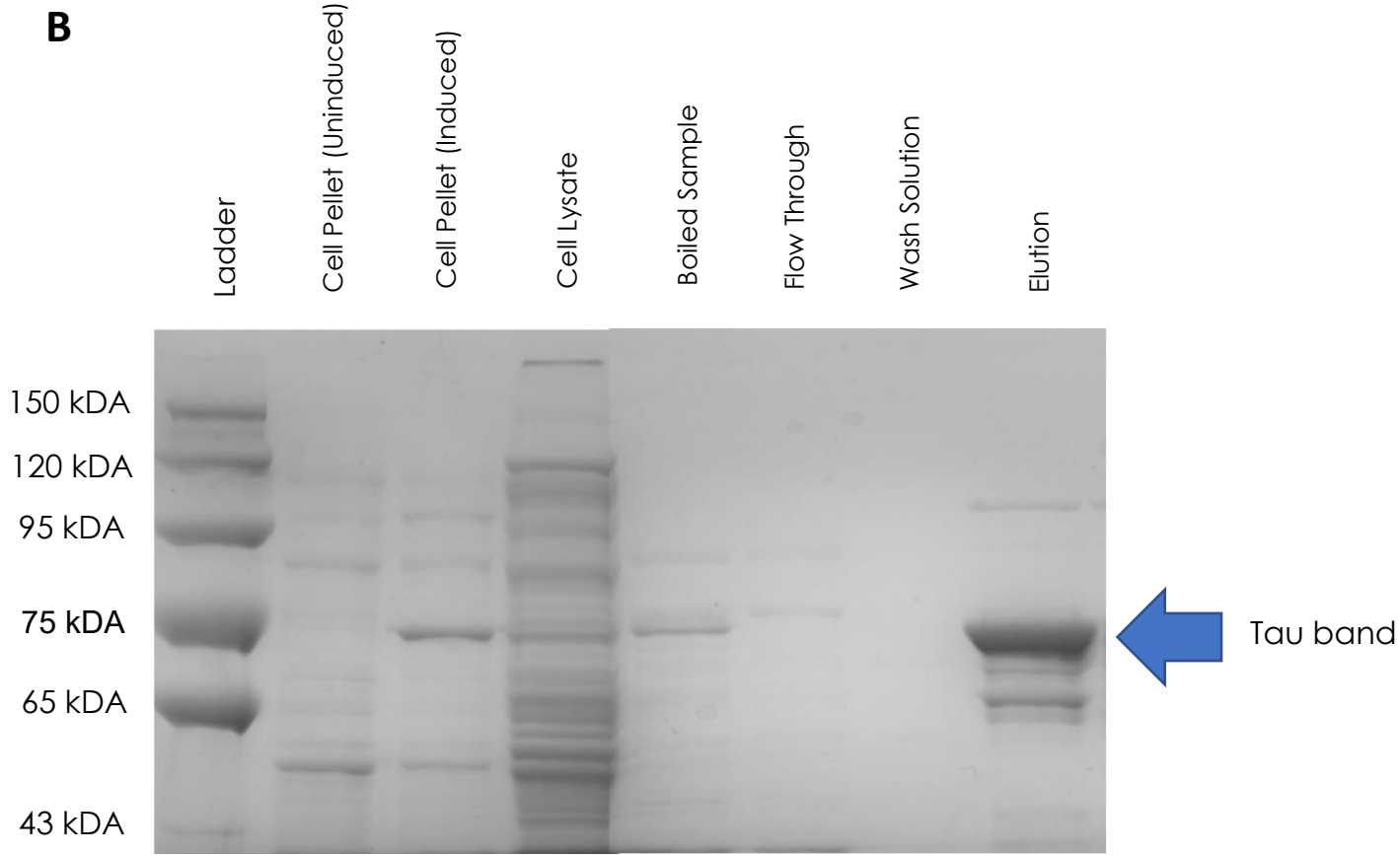
Figure 2: Purification of Tau/Acetylated-Tau from *E. coli*. A. Schematic Diagram of Purification Process Schematic showing pictures from each step of nickel purification. Displaying cells post lysis, post boiling, nickel affinity column, and protein in concentrator tube. **B. Purification of 4L Induction of FLT.** 8 μl of sample from various stages of nickel affinity chromatography purification were ran on 8% SDS PAGE and visualized with Coomassie blue staining. **C. purification of 4L Induction of AT.** Like B except A-Tau production is being induced

Legend: ● Absorbance (mAu)

● % Solution B

A
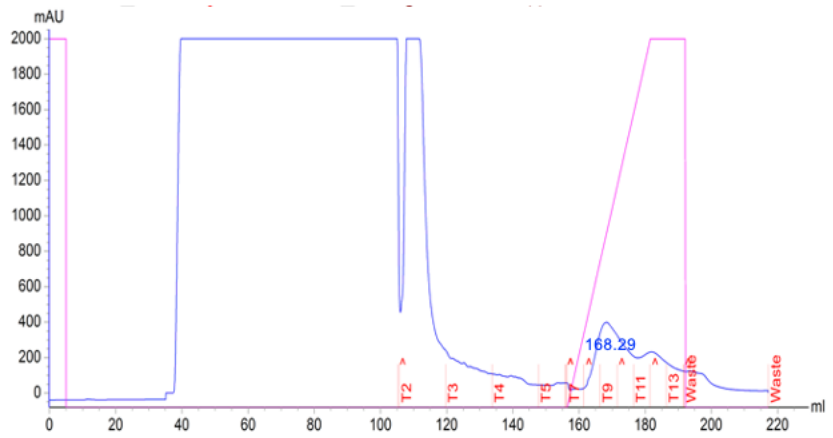
No	Peak name	Retention ml	Area ml*mAU	Height mAU
1	Tau Fractions	168.29	3733.4980	368.854

B
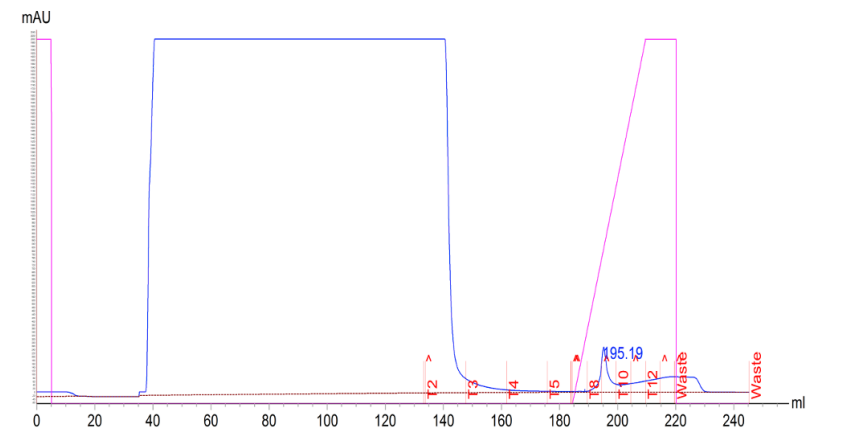
No	Peak name	Retention ml	Area ml*mAU	Height mAU
1	A-Tau Fractions	195.19	868.6492	256.610

Figure 3: Chromatograph from FPLC Purification of Tau Pellets A. Chromatograph from an FPLC Nickle Purification of 4L of FL Tau Approximately 60 mL of protein rich fluid acquired from 4L induction was ran on FPLC column. Weakly binding protein was washed off column with low salt buffer. Elution of protein bound to column occurred on increasing imidazole gradient with initially none in solution until 500 mM imidazole was reached. Fraction collection was automated and fractions 8-11 were collected for future experiments. Relevant peaks were integrated. **B. Chromatograph from a Nickle Purification of 4L of A-Tau**. A chromatograph from a 4L induction of A-Tau. Like D, but from an A-Tau preparation.

A

Ladder

Full Length Tau

Full length Tau

Concentrated protein

150 kDA

120 kDA

95 kDA

75 kDA

65 kDA

43 kDA

B

Ladder

Frozen in 10% glycerol

Frozen in 10% glycerol

Frozen in liquid N_2

Frozen in liquid N_2

Precipitated and resuspended

Precipitated and resuspended

-20^0C

-20^0C

4^0C

4^0C

150 kDA

120 kDA

95 kDA

75 kDa

65 kDA

43 kDA

25 kDA

Tau band
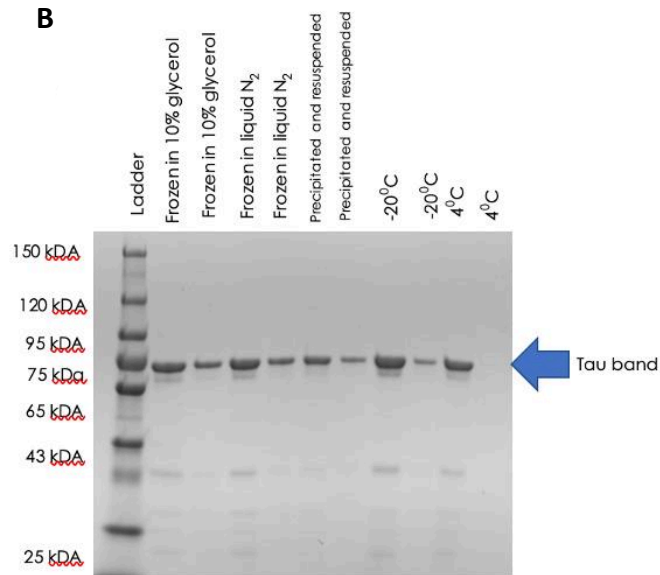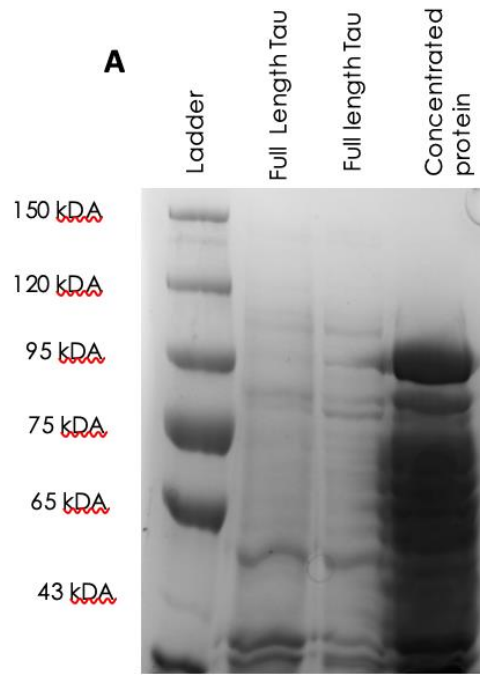
Figure 4: Determining optimal storage conditions for purified Tau. A. Concentrating Eluted Protein Purified Tau samples that had a concentration less than 5 mg/ml were concentrated overnight and 8 μl of each sample was ran an on 8% SDS PAGE and visualized with Coomassie blue staining. **B. Protein Storage Stress Test** Tau aliquots were kept in outlined conditions over 4 days and 8 μl was ran on 4-20% SDS PAGE visualized by Coomassie blue staining. Samples were run at a 1:10 and then 1:20 dilution. Lanes 1-6 were stored at -80^0C

A

Blank Tau

⟵ Tau band

B

Ladder Full Length Tau 5.0 mg/ml Full Length Tau 2.5 mg/ml Full Length Tau 1.3 mg/ml

⟵ Tau band

C

Ladder

Tau

Oligomers

Monomer

D

Ladder

Tau

Dimer

Monomer
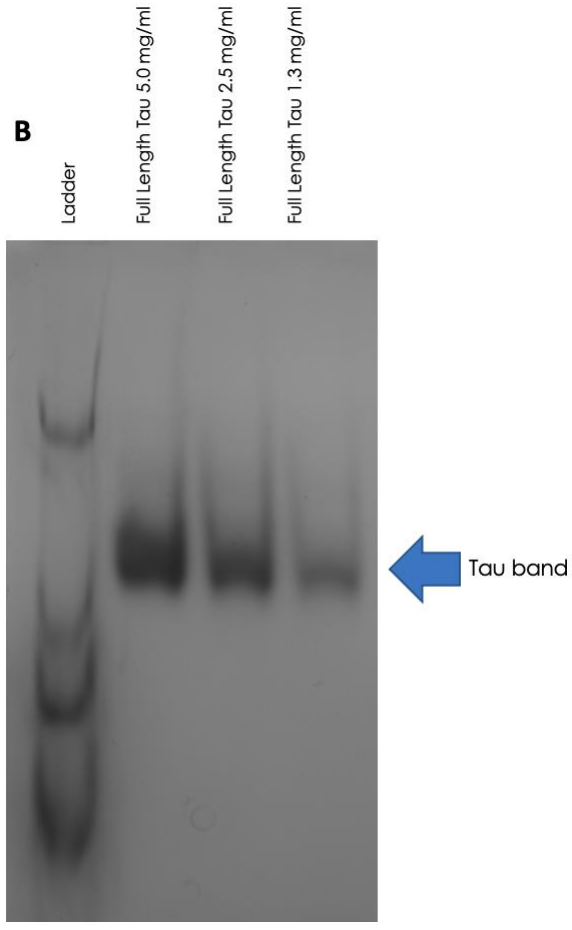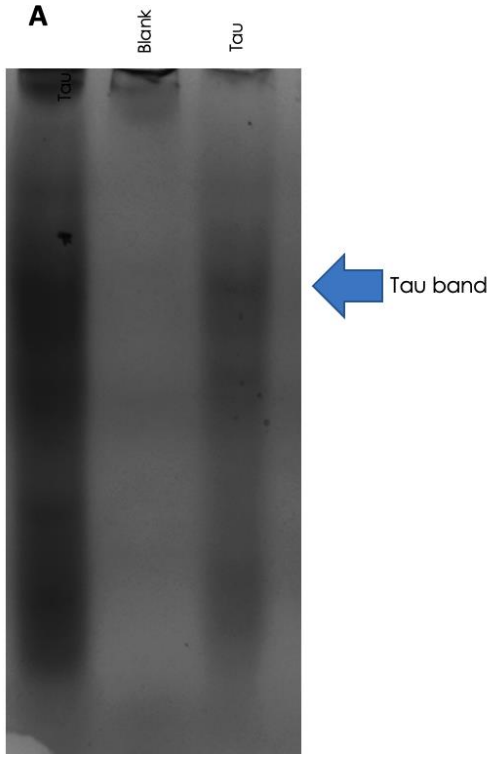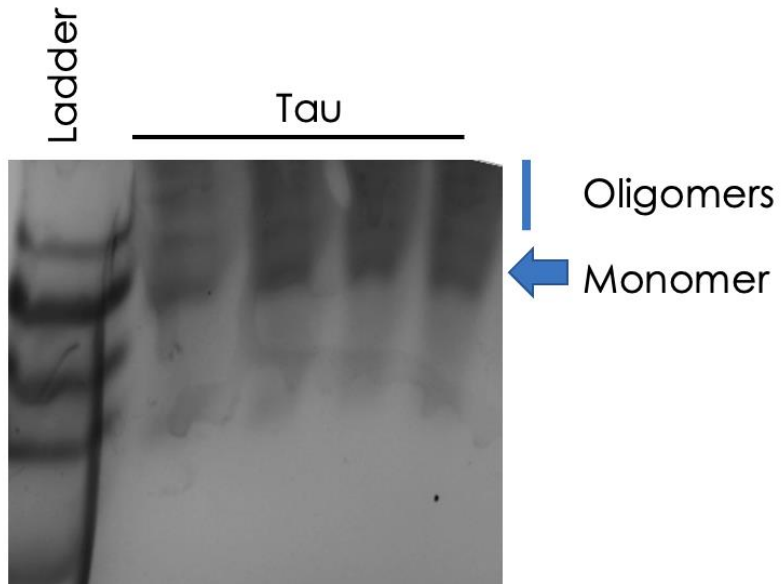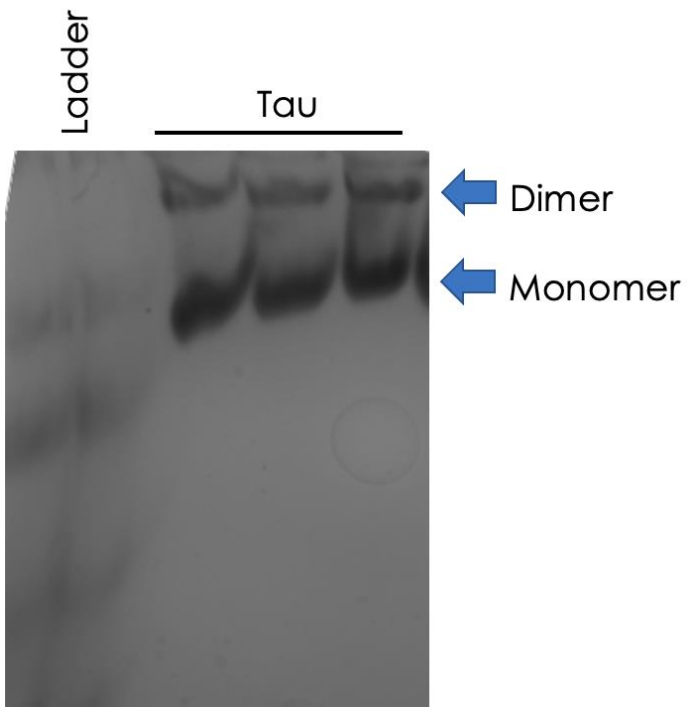
Figure 5: Optimizing Native PAGE Conditions to Visualize Tau Oligomers. **B. 5% Acrylamide Native PAGE** 5% Native PAGE of 5 mg/mL visualized using Coomassie blue. 15 µl of each sample was ran in each lane. **B. 5% Acrylamide Native PAGE 5**% Native PAGE of of serially diluted Fl-Tau Fl-Tau visualized using Coomassie blue. 15 µl of each sample was ran **C. 10% Acrylamide Native PAGE** 10% Native PAGE of 2.5 mg/mL Fl-Tau visualized using Coomassie blue. 15 µl of each sample was ran in each lane **D. 10% Acrylamide Native Page Ran at Constant Amperage** 10% Native PAGE of 2.5 mg/mL Fl-Tau visualized using Coomassie blue. 15 µl of each sample was loaded, gel was run with updated protocol.

A

Ladder

Tau

← a-Tau

B

Ladder

Tau

← a-Tau

31
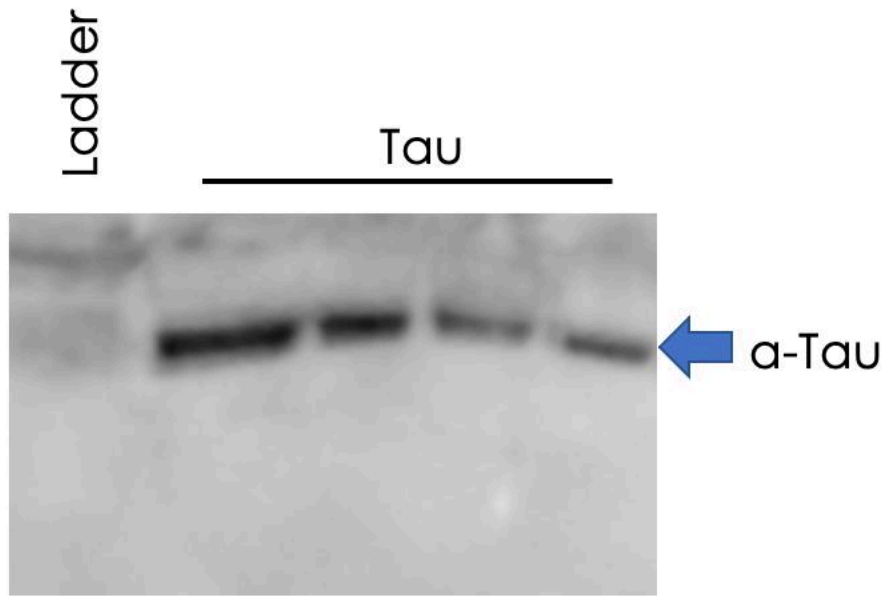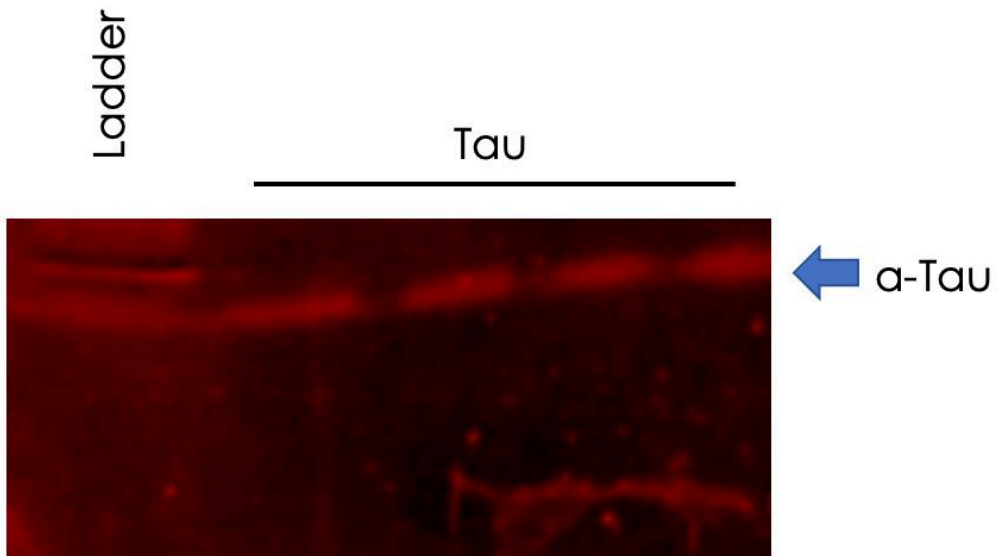
Figure 6: Western Blots of Purified Tau Samples A. Chemiluminescent Western Blot of Tau 10% SDS PAGE of Tau was ran and transferred to low-fluorescence PVDF membrane. Membrane was tagged with HRP and ECL reagent was applied to membrane before imaging. **B. Fluorescent Western Blot of Tau** 10% SDS PAGE was ran and gel was transferred to low-fluorescence PVDF membrane. Membrane was tagged with Fluorescent secondary antibody and imaged.

Discussion

Producing Post-Translationally Modified Protein in a Bacterial Expression System

Bacterial expression systems are a popular choice for recombinant protein production. Bacteria are easy to grow, allow for high yield, and are relatively in expensive. A continually replenishing culture of stock bacteria for protein production can be modified to create a protein of interest through transformation of a plasmid that contains the genetic information necessary to create it. When undergoing environmental stress such as heat or electricity, a bacteria's cell membrane becomes porous, and it can intake foreign DNA from its environment. However, although bacteria can produce protein from recombinant DNA and create protein that they normally cannot, they are unable to perform eukaryotic specific post-translational modifications. Bacteria do not innately possess the necessary eukaryotic enzymes to post-translationally modify a protein at a rate that is relevant for research (Macek et al. 2019). However, with the introduction of a second plasmid the bacteria can then modify protein at a level where experiments can be done. This second plasmid allows the bacteria to create N-terminal Acetyltransferases (NATs), which catalyze the addition of an acetyl group to the N-terminus of Tau co-translationally (Starheim et al. 2012).

Although bacteria have short generation times and will quickly dispose of any DNA that is deemed unnecessary for survival because of the error prone nature of their replication, (Tippin et al. 2004), controlling for bacteria that have both plasmids present is a simple procedure. Each plasmid contains an antibiotic resistance gene that allows bacteria to grow in selective culture. In a double transformation system, each plasmid contains a different antibiotic resistance gene, and a bacteria must keep both to be able to survive in the selective media that it is placed in. However, the increased amount of antibiotics appears to result in slower bacterial growth and less total protein production when compared to a single transformation system.

The flexibility of this system allows for the introduction of one plasmid for production of unmodified Tau or a second plasmid to create the acetylated form of it. Also, because the production of protein is inducible through a modified lac operon instead of being constantly active in this system, the bacteria can grow normally until reaching a suitable concentration of protein production. Production is induced by Isopropyl β- d-1-thiogalactopyranoside (IPTG), a mimic of allolactose that bacteria are unable to metabolize and as a result concentrations in culture remain constant.

Isolating Protein from Cell Lysates

When implementing a bacterial expression system, the protein that is produced must be isolated from bacterial cell lysates and culture. Although this may not be necessary when performing Western blots, testing for protein expression, or ligand binding studies, structural work on protein requires pure samples. While Tau does not have a fixed 3D structure, NMR (Nuclear Magnetic Resonance) can allow some visualization of its 3D structure (Gibbs et al. 2017).

Because Tau is an intrinsically disordered protein, it can withstand conditions that cause other proteins to denature. This allows us to boil our whole cell lysates to cause other bacterial proteins to precipitate out of solution, and to deactivate bacterial proteases that may degrade our protein (Livernois et al. 2009). Tau remains soluble in solution, and we can move onto nickel affinity chromatography to further purify our sample. Tau expressed by the bacteria has a polyhistidine tag on its C-terminus, a motif that allows the protein to be separated from whole cell lysates by granting the protein a high affinity to nickel. By passing our boiled samples in a high-pressure system through a nickel column, we can bind Tau to the

column. Proteins lacking the poly-histidine tag will be unable to bind to the column and will simply pass through the column. Following this, we can pass a solution of imidazole (the functional group of histidine) through the column to dislodge the Tau and collect a pure sample of the protein. Nickel affinity chromatography shows good yield of protein, and results in relatively pure samples (Bornhorst and Falke 2010). The poly-histidine tag is placed on the C-terminus of a protein because proteins are normally translated from N-terminus to C-terminus, so protein with high affinity to the column should be complete Tau portions.

When attempting to perform experiments involving protein one major concern involves the storage, purification, and other miscellaneous processing of the protein. Although Tau is an intrinsically disordered protein and is more stable at room temperature than other proteins because there is no worry about loss of 3D structure (Uversky 2015), it can still undergo degradation if kept in harsh conditions or freeze-thawed excessively. Because of this, proper storage of the protein is integral to performing future experiments. It is typical to store protein in a –80°C freezer for long term storage but freezing the protein while minimizing degradation is an issue.

Acetylated Tau in particular produces an elutant that is too low in concentration to be able to do many experiments with. Although theoretically larger scale inductions could be done it is unclear if this would increase the protein concentration to a usable level. Proteins were instead concentrated using a centrifuge tube with a polyethersulfone (PES membrane that retains molecules of a certain molecular weight/ Proteins were concentrated to 5 mg/mL, divided into 100 μL aliquots and frozen for future experiments.

Visualizing Protein

After production of the protein, a key step in experiments is simply to visualize the protein that has been produced. Because we are attempting to look at the effect of a post-translational modification of a protein or the oligomerization state of the protein, two obvious choices for visualization are Western blotting and native PAGE.

Western blots are a powerful method of being able to view protein. It is an immunoassay detecting protein that has been fixed onto a membrane with the two antibodies and is highly specific in nature. The first antibody to bind is the primary antibody. Primary antibodies are capable of binding to specific proteins that are in different physical states, such as selectively binding to either acetylated or unacetylated Tau (Saper 2009). Secondary antibodies then go on to bind to these primary antibodies and allow us to visualize our samples using either chemiluminescence or fluorescence. Although the SDS-PAGE that the Western blots were taken from did not run evenly, it is unimportant for the visualization of the protein. The increased resolution from a cleanly running PAGE was not relevant, as any signal shows successful secondary antibody binding and indicates a successful Western blot.

Native PAGE is another method in visualizing protein that has been produced. This is a non-denaturing gel that allows for separation of a protein based off of its mass, charge, and structure. While developing the protocol for native PAGE, many factors were changed about the composition of the gel and the running conditions. Gel acrylamide percentage is one such factor. Increasing acrylamide percentages decreases pore size after casting and allows for better visualization of small proteins because larger proteins become stuck in the smaller pores. 10% acrylamide concentration appears to give acceptable resolution. Another factor that was viewed was with the decision to either make the voltage or the amperage while running the gel the constant. Although granting good resolution, the heat from running a gel at constant amperage results in uneven running and makes interpreting results difficult, protein was able to be visualized on the gel.

Future Directions

Now that there is a rudimentary understanding of the conditions that can be used to run a native PAGE and even the presence of some high running bands that are possibly species of Tau that have spontaneously aggregated in solution, there is the possibility of performing blue native PAGE. Because we are seeing some bands that are running higher than the most concentrated bands in our sample, there is reason to believe that these bands are higher-ordered structures. The only protein that is being ran in high enough concentration to be visualized on the native PAGE is Tau, and Tau has been known to spontaneously aggregate in solution. Although further confirmation is needed, it seems that it is possible to view aggregates with native PAGE. Blue native PAGE is a non-denaturing method of separating protein, there exists a variation of the method that allows for observation of protein-protein dynamics and separation of protein based on their oligomerization state (Witting 2006). This method of detection would allow for definitive identification of the higher running bands and would allow us to use blue native PAGE as a method to quantify aggregation.

Western blotting allows for selective assessment of protein samples. Fluorescent staining is the avenue to which the protocol is being moved to. Chemiluminescence relies on an enzyme-substrate reaction to produce light and because of this is hindered due to the varying rates of reaction kinetics, not allowing for a quantitative analysis of protein in solution. This makes it useful for a very sensitive method of protein identification but limits its other applications. Fluorescence on the other hand is directly proportional to the total amount of protein that is present on the gel. This allows for fully quantitative calculations done with the amount of light emitted, and because each antibody emits light in a specific wavelength multiple antibodies can be used for complex assays (Gingrich et al. 2000). These assays can look at acetylated vs unacetylated ratios in human neuronal tissue to see acetylation rates in patients suffering from ND.

While the protein samples that are produced from the nickel affinity chromatography are relatively pure, to perform high resolution structural analysis of a protein such as with x-ray crystallography protein purity should reach at least 95% (Kim et al. 2009). Although it is possible to simply pass the protein through the nickel column an additional time to purify it further, proteins that bind non-specifically to the nickel column may be impossible to remove from solution. Other forms of chromatography may be employed to narrow down conditions to a point where Tau is more concentrated in solution. Ion exchange chromatography (IEX) is one such method of separating proteins based on another physical characteristic. IEX instead uses a protein's net surface charge and affinity to an ionic column to separate it from solution (Jungbauer and Hahn 2009). Using an ionic column instead of a nickel column, and a pH gradient instead of an imidazole gradient, the protocol for the nickel purification can be easily adapted to this new method. Gel filtration chromatography is another chromatography method that can separate proteins in solution. Instead of further purification, this allows for the separation of proteins based off their molecular weights (Hagel 2001). Concentrations of higher-order structures such as dimers, trimers, or oligomers can be viewed, and aggregation rate of Tau can be assessed.

Having a reproducible method to create large amount of Tau in both its acetylated and unacetylated state, it is possible to do experiments that are concentration dependent. Previously experiments involving liquid-liquid phase separation or aggregation were not possible because they are concentration dependent (Franzmann and Alberti 2019), but with revisions to our protocol it is now possible to use these experiments. Although much of the work that was done was simply in the production and purification of the protein, this is integral to doing more advanced analytical studies with the protein in the future. NMR and X-ray crystallography require protein in high concentrations and pure samples of protein (Kim et al. 2009), so structural assessments of the protein in the future need methods such as these.

If something such as the ability of acetyltransferase to consistently modify protein is affected with age, this would be a relevant avenue of research. Being able to determine if this decrease in acetylation rate is an early marker for a Tauopathy could be something that can be used in counseling to begin prophylactic treatment, or if an individual is known to have some sort of family history of neurodegenerative Tauopathies that they could be administered a drug to assist in acetylation.

Tau aggregation remains a mysterious part of Tauopathies and other diseases that the protein is involved in, so further characterization of the structure, the effect that N-terminal acetylation has on the protein, and how it can interact with itself in solution is critical when considering new effective methods to combat these illnesses.

References

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