EFFECT OF TAU HYPERPHOSPHORYLATION ON CELLULAR PATHOLOGY

by

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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Hyperphosphorylation of the microtubule associated protein tau is shown to be involved in several dementias that classify as tauopathies. In these diseases, tau is known to bind to itself rather than associate with microtubules. When CHO cells express wild type tau, the microtubule network is healthy and shows normal microtubule movement and tau associated with the microtubules. When expressing pathological human tau (PH-tau, pseudophosphorylated at T212, T231, S262),however, PH-tau is present throughout the cytoplasm, rather than associated with microtubules. The cells exhibit excessive membrane blebbing in order to remove PH-tau. This blebbing leads to a shrinkage of PH-tau expressing cells. Internally the presence of excessive cytoplasmic vacuoles and aggregated PH-tau in the form of filaments are found. The exposure of wild type expressing cells to okadaic acid shows the same pathologies. Additionally, all three sites used in the PH-tau construct are phosphorylated when wild type tau is exposed to okadaic acid.

Tau interacts with actin as well as with microtubules. PH-tau seems to cause a major breakdown in the F-actin structure within the cells. These cells appear to be either totally void of F-actin or have F-actin forming punctate spots within the cells. This actin breakdown is also occurs in wild type tau expressing cells treated with okadaic acid. The CUNY CSI Computer Science Department is working in collaboration to develop ImageJ plugins to quantify the amount of F-actin and the length of individual F-actin filaments. This work demonstrates that when PH-tau is expressed, the level of tau is inversely proportional to the level of F-actin in the cells. Interestingly, the level of total actin does not change between wild type tau and PH-tau expressing cells, suggesting that this lack of F-actin does not change expression, but rather interferes with its polymerization.

When CHO cells are transfected with PH-tau, this protein can be found in the nucleus of the cells. We found a nuclear localization signal that allows the chaperon protein importin to bind to tau. To see if this site was responsible for the translocation of tau into the nucleus, we eliminated importin's binding site through site directed mutagenesis of the full-length tau gene. The elimination of the importin binding site inhibited tau from being able to translocate into the nucleus but did not stop any of the pathologies seen previously.

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This thesis if dedicated to my parents,

Stephan and Kathleen Corbo

I cannot thank you both enough

Mom, we miss you

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ABBREVIATIONS

AD	Alzheimer's Disease
WT-tau	Wild type tau
PH-tau	Pathological Human Tau
MAP	Microtubule Associated Protein
NLS	Nuclear Localization Signal
FTDP-17	Frontal Temporal Dementia with Parkinsonism linked to chromosome 17
CNS	Central Nervous System
CHO	Chinese Hamster Ovarian Fibroblasts
TEM	Transmission Electron Microscope
SEM	Scanning Electron Microscope

CHAPTER ONE

Background and Significance

Published Work:

Therapeutic targets in Alzheimer disease and related tauopathies
Christopher P. Corbo and Alejandra del C. Alonso
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Abstract: Alzheimer's disease is a progressive neurodegenerative disease that is characterized histopathologically by the presence of plaques, mainly composed of Abeta amyloid and the tangles, mainly composed of hyperphosphorylated tau. To date, there is no treatment that can reverse the disease, and all the current therapeutics is directed to cope with the symptoms of the disease. Here we describe the efforts dedicated to attack the plaques and, in more detail, the process of neurofibrillary degeneration, linked to the presence of the hyperphosphorylated microtubule associated protein tau. We have identified the different putative targets for therapeutics and the current knowledge on them.

1.1 - INTRODUCTION TO THE DISEASE

1.1.1 - History

Alzheimer's disease is a neurodegenerative disorder causing dementia in affected patients. Alzheimer's disease is distinguished by the characteristic senile plaques and neurofibrilary tangles seen in the cortical tissue of post mortem brains.

Alois Alzheimer first characterized Alzheimer's disease in 1906 based on pathology he classified from the post-mortem brain of a senile patient. Alzheimer's disease and other similar dementias are characterized by neuronal loss beginning in the entorhinal cortex of the hippocampus and progressing further. In late stages, the brain will be reduced in size by up to 25% in cortical regions (Frisoni et al., 2007).

Alois Alzheimer was a German trained physician beginning his medical education at the University of Berlin, later transferring to the university of Wurzburg under the supervision of Dr. Kolliker under whom he learned a great deal of histological preparation and microscopic analysis. After Alzheimer completed his studies, he received a position as an Assistant Professor at the Municipal Asylum for the Insane and Epileptic in Frankfurt. It was here that Alzheimer met Franz Nissel, whom he worked with very closely. Together, the two scientists with their supervisor Emil Stoli, transformed the institute into not only a housing facility for the patients, but also an acceptable clinical center focusing on disease research and possible treatments. Alzheimer soon became known as the "psychiatrist with a microscope," as he correlated behavioral issues to neuropathology. In 1902 he moved to Heidelberg and then to the Cerebral Anatomical Laboratory in Munich, working with Emil Kraepelin at both places. It was during his years in Munich that Alzheimer found the pathological lesions associated with

Alzheimer's disease today. Finally, he was appointed as a Professor of Pyschiatry at the University of Breslau in 1912 and died at 51 years of age in 1915 (paragraph cites Perry et al, 2006).

Alzheimer's disease did not become a major health concern until the 1960s. This is because in earlier years, the life expectancy was not very high. In other words, people did not live long enough to become senile. It was becoming very clear that Alzheimer's disease did not only effect by individual who has the disorder, but also takes a large toll on the family and caretakers of the individuals. As the number of patients with this disorder increased, there became a need for further research into the underlying mechanisms as well as work towards the development of a treatment.

Early work focused furthering the work of Alzheimer looking into the neuropathological features of the disease. The electron microscope allowed for the identification of the sub-cellular structure of the amyloid plaques and helped to lead toward the identification of amyloid beta involvement (Kidd, 1963).

With the discovery of genes and inheritability and the advancement of the necessary techniques to study genes, genetic factors became an interesting approach. In the 1990s, several genes were discovered with implication in early-onset Alzheimer's disease, which is Alzheimer's disease that occurs in multiple individuals in one family line. This type of the disorder has a very early onset, mid to late forties as opposed to late sixties early seventies. The key genes discovered are apolipoprotein E(ApoE) (Strittmatter et al., 1993), amyloid b-protein precursor (APP) gene (Goate et al., 1991), presenilin 1 (PS1) gene (Sherrington et al., 1995), and presenilin 2 (PS2) (Levy-Lahad et

al., 1995). All of these genes with the exception of the ApoE are implicated in early onset Alzheimer's disease. ApoE gene is seen in roughly half the Alzheimer's patients, but it is not deemed a marker for the disease since it seems more a risk factor than a cause. The APP gene was the first discovered due to its relation to the production of amyloid beta. Prensenilin 1 and 2 mutation occur in 50% of the Alzheimer's patients and there have been a number of mutations identified, but there are many situations where a family posses a mutation that is not seen in any other pedigree, so it is hard to correlate the gene mutations to the disorder (Blacker and Tanzi, 1998). Locating genes implicated in late onset Alzheimer's disease has had been quite difficult until the development of the large-scale genetic screen. Pericak-Vance et al(Pericak-Vance et al., 2000) were able to screen many patients and begin the work into finding similarities in genetic mutations within late onset Alzheimer's disease patients. Further work is being pursued in this area.

Early treatments for Alzheimer's disease focused on replenishing acetylcholine in the brain. The first drug treatments used were food supplements that contained acetylcholine precursors, choline and lectine for example (Amenta et al., 2001). These trails met little success. Further work focused on tacrine, a drug used to counter scopeolamine, a common anesthetic. The initial results by were promising, but soon discontinued due to an increase in transaminases leading to liver dysfunction. Acetylcholineesterase inhibitors were another focus such as donepizil. Drugs such as these are able to treat symptoms for a period of time, but do not slow the progression of the disease. Further work focusing on the specific molecular mechanisms of Alzheimer's disease are being looked into and are the primary focus of this review (Perry et al., 2006).

1.1.2 - Symptoms and Stages

The symptoms of Alzheimer's disease align with many other types of dementia in that the effected individual has problems with the way in which their brain processes and recalls information as well as how the individual is perceived by others. Major changes in a person's demeanor are detectable in that the patient seems like a different person to those who have known them for an extended period of time. Alzheimer's disease also alters a person's ability to perform routine daily tasks, like washing and dressing and maintaining a household.

The disease is categorized into seven stages (as characterized by Barry Reisberg, M.D., Clinical Director of the New York University School of Medicine's Silberstein Aging and Dementia Research Center). The stages described here are adopted from those listed by the Alzheimer's Foundation (www.alz.org).

The first stage of the disease is normal function. At this stage, there are no memory loss or personality changes. Stage two seems much like what one would classify as normal aging, in that they forget names occasionally and loose their eyeglasses or keys. At this stage, it is not yet possible to state that a person has Alzheimer's disease, or is just presenting normal aging characteristics. At Stage three, the forgetfulness becomes more apparent. At this stage, word and name forgetting increases and more valuable objects are misplaced and reading comprehension is lost. There is also a noticeable decline in the person's ability to plan out events.

At stage four, the patient is considered to be in early to mid-stage Alzheimer's disease. There are clear cut deficiencies in their memory and their ability to plan and

carry out daily activities. At this stage, all of the problems are noticeable in a psychiatric medical exam. They are unable to perform basic mental arithmetic and reading comprehension tasks. They also now display a reduced knowledge of personal history. At stage five, patients are often at this time required to have some home care since they are not able to manage required skills. At this point, patients are often forgetting basic information such as their address and telephone numbers as well as date, month and season which they are in. At stage six, the mental capacities continue to decline, but physical problems begin to occur such as problems controlling bowel movements and urination. Daily events are often not remembered by the patient as well. At this point, they forget the name of their spouse or daily caregiver as well. By stage seven, the patient is usually hospitalized requiring constant care with all activities such as eating and using the restroom. At this stage, the patient often will loose their ability of speech and will not be able to walk.

1.1.3 - Pathology

The neuropathology of Alzheimer’s disease consists of two protein aggregates, one being the aggregation of amyloid beta into what is termed plaques. The other is the aggregation of hyperphosphorylated tau into tangles. It has been believed that these neuropathological states cause the massive neuronal loss and the brain inflammation that is classically seen in post mortem Alzheimer disease brains.

Amyloid beta is a fragment of the larger amyloid precursor protein (APP) after it has been cleaved by beta and gamma secretases. APP is an integral membrane protein that is highly concentrated in the synaptic membrane and is believed to play a role in

synapse formation and synaptic plasticity (Turner et al., 2003). When cleaved by gamma secreatase, various size fragments of beta amyloid are released from the membrane. The fragments most frequently seen are Abeta1-40 and Abeta 1-42. These fragments easily associate with one another and aggregate in the brain to form the plaques, which are composed of 6-10 nm long fibrils associated with one another. This is the original pathological lesion documented by Alois Alzheimer. In order to balance the production of these Abeta fragments and their further degradation in order to avoid buildup, the brain has several other proteases that are activated by several different pathways and cleave the fragments at several different sites. Such examples are plasmin, neprilysin, and insulin degrading enzyme (De Kimpe and Scheper, 2010).

For an unknown reason, the amyloid beta is not further degraded and consequently accumulates in the brain. This remaining protein is toxic to neurons and several mechanisms of toxicity have been studied. It has been seen that amyloid beta causes physiological damage to neurons as well as causing an inflammatory response from surrounding glial cells (Salminen et al., 2009).

The second classical lesion seen in Alzheimer's disease is caused by the hyperphosphorylation of the microtubule associated protein tau (Grundke-Iqbal et al., 1986b). Microtubule associated proteins (MAPs) interact with tubulin to stabilize microtubules. Tau is the MAP associated specifically with the brain. The collective group of neurodegenerative diseases associated with tau hyperphosphorylation are commonly referred to as tauopathies. These diseases are histopathologically characterized by neurofibrillary tangles (NFTs) composed of hyperphosphorylated forms of tau. Normally this phosphoprotein plays a key role in the stabilization of the microtubule networks.

When hyperphosphorylated, tau no longer binds to microtubules and begins to aggregate. This aggregation is detrimental to the cell in two ways; first the cell is not able to maintain their axons due to a lack of sub cellular movement. The networks of microtubules that compose the axon are critical in the movement of specific neurotransmitters and other vesicles as well as mitochondria for energy production to the synaptic cleft. Secondly, the tau aggregates are also neurotoxic as they begin to occupy large regions of the soma and leave little space for normal cellular functions to occur. Further information on tau pathology will be covered later in the article.

1.1.4 - Animal Models

As is the case in most biomedical research, a well-characterized animal model is imperative for the progression of research into possible treatments for the disorder. There have been several models developed for Alzheimer's disease and Frontal Temporal Dementia (FTD). Most of the models have centered around mouse models, but there have been models developed in organisms such as Drosophila melanogaster (Feuillette et al., 2010), Caenorhabditis elegans (Ewald and Li, 2010), and Danio rerio (Xia, 2010). Most of the successful models focused on transgenic approaches but a few chemically induced models have been developed, such as the use of okadaic acid (Wang et al., 2001). The chemical models lack the classical pathological lesions normally seen in Alzheimer's disease.

Representative mouse strains expressing amyloid precursor protein mutations are J20, Tg2576, PDAPP, and APP23 (Gotz and Gotz, 2009). These strains are well

characterized and have robust amyloid precursor protein and amyloid beta pathology (Gotz and Gotz, 2009).

Transgenic mouse models expressing tau mutations include P301L mice. In this system, calcium calmodulin dependent protein kinase II driven promoter was used in order to facilitate the production of ten time more mutant tau than endogenous tau (Ramsden et al., 2005). In order to see the effect of these mutations, a large amount of mutant protein must have been produced as the endogenous tau within the mouse proved to interfere with the system. For this reason a knock out of endogenous mouse tau and a knock in of mutated tau proved more effective (Ramsden et al., 2005).

Transgenic models looking into the various sites of phosphorylation on tau have been conducted in flies. It has been shown that with up to 14 sites pseudo-phosphorylated, the flies died earlier than those expressing wild-type tau (Alonso et al., 2010; Steinhilb et al., 2007a). It was demonstrated that the phosphorylation sites work in conjunction to yield toxicity rather than independently when mixing the number of sites pseudo-phosphorylated to those in which alanine was the switched in amino acid(Steinhilb et al., 2007a).

When targeting possible therapies, these mouse strains have proved useful as they provide a platform for the testing of therapeutic agents on the various targets of Alzheimer's disease pathology. Further information discussing the use of these models will be addressed in the next section of this paper.

1.1.5 Biomarkers

A major concern with Alzheimer's disease is the difficulty in diagnosis. Since the markers of the disease are pathologically only seen in the brain tissue and are regions where biopsy is not possible, the only way to diagnose the disorder is by psychological evaluation. Looking for a biomarker for Alzheimer's disease is of great interest to the field of Alzheimer's disease research.

The approach to discover a marker for Alzheimer's disease is taking two paths, one looking for a biochemical based marker from a particular, easily accessible fluid such as blood or cerebro-spinal fluid (CSF). Another approach is looking for imaging markers utilizing volumetric magnetic resonance imaging scanning (vMRI) and positron emission tomography (PET) systems. The imaging approach has been further developed, but due to the timing and the cost, is slow to generate enough data in order to make the proper correlations. There have been studies looking into the size of regions such as the entorhinal cortex and correlating size with the disease progression and there were correlations but further work is needed (Thal, 2006). Biochemcial markers would provide to be easier to acquire and process. There have also been studied on both Abeta and hyperphosphorylated tau detection in both CSF and blood plasma. When looking into Abeta in CSF, the levels do correlate to the level of dementia as well as to aged matched controls, but the levels were mostly stable during the course of the experiment. When looking at the levels of Abeta 42 in blood plasma, the levels did not correlate the way the CSF studied did. The other pathological biomarkers, tau, was also analyzed in CSF. The levels of tau in the CSF was correlated to the dementia scores, but much like the Abeta analysis, the levels did not change much over the course of the 12 month study (Thal,

2006). Without the ability to detect changes in levels are compare them to the behavior of the individual the biomarkers is not a reliable measure.

The push to find a biomarker is not primarily for diagnosis, but more for clinical trials, in which certain treatments can be tested and there is a strong finite measure of the effect of the treatment. Without such a marker, the development of a successful treatment will be difficult and time consuming as the best measure at this time is psychological evaluation.

1.2 - ATTEMPTS AT Aβ THERAPY

1.2.1 - Antibody Therapy

It has been shown that there is a potential for treatment of Alzheimer's disease with antibody therapy. In 1999, Schenk (Schenk et al., 1999) and colleagues were able to immunize PDAPP mice against amyloid beta and were able to see that present plaques could be broken down and future deposits were greatly reduced . Furthermore, these mice had improvements in their cognitive function. These positive results lead to a clinical trial that was stopped due to adverse side effects in the patients (Dodel et al., 2010).

Several experiments have shown a decrease in deposited amyloid beta. These experiments were carried out primarily in animal models expressing APP mutations. One such experiment utilized the activation of microglial cells via the complement system. Antibodies can activate the complement system via an Fc-dependent (Bard et al., 2000) or an Fc-independent pathway (Das et al., 2003) and leading to phagocytosis of the plaques.

There are several mechanisms of antibody therapy that has been looked into. Catalytic antibodies will bind to the amyloid beta and render it non toxic(Taguchi et al., 2008). A second mechanism has been using conformational specific antibodies, which bind to and change the conformation of the protein to make, either aggregated or lose, in order to make it less prone to bind. These studies have been conducted *in vitro* (Bacskai et al., 2001).

1.2.2 - Secratase inhibitors

Several membrane bound secretase enzymes are responsible for the cleavage of amyloid precursor protein (APP) into the its various amyloid fragments, including the amyloid beta fragment which is the primary component of the senile plaques, one of the major pathological lesions seen in Alzheimer's Disease. Certain secretases have been considered possible therapeutic targets against the production of amyloid beta.

Alpha secretase is the enzyme that cleaves APP outside of the CNS (Seubert et al., 1993). The interest in this secretase as a possible therapeutic target is in its ability to cleave APP in between amino acid resides 16 and 17, therefore generating fragments that are soluble and easily broken down (De Strooper et al.). Alpha secretase in the brain would be neuroprotective since it would facilitate the production of soluble p3 molecules via the further breakdown of the amyloid sequences by gamma secretase (Haass and Selkoe, 1993). The issues with using this particular secretase is that it is controlled by several proteases including disintegrin and metalloprotease (ADAM), which make direct manipulation of alpha secretase difficult. Furthermore, analysis of the role of these

proteases has proved difficult since animals die very early in gene deletion studies (Hartmann et al., 2002; Maretzky et al., 2005). The possibility of alpha secretase as a possible therapeutic target would be enhanced with the proper animal models in order to determine which ADAM protease is most effective in cleaving APP (De Strooper et al., 2010).

Beta secretase is another secretase that has been a possible target for Alzheimer's disease therapy since this is the enzyme that cleaves APP into the 42 amino acid Abeta sequence (Vassar et al., 1999). Specifically, the beta secretase BASE1 has been identified as the primary enzyme present in the brain facilitating amyloid beta production. Transgenic mice without BASE1 have been bred with APP mutant mice and it has been seen that there is far less amyloid beta present in these animals(Cai et al., 2001; Luo et al., 2001). Through these experiments, it has become evident that there are physiological deficits in these mice, although they are able reach adulthood and successfully breed. These other physiological conditions raise concerns as to how well this particular enzyme will be as a therapeutic agent since further research into what other reactions this particular enzyme is involved in (De Strooper et al., 2010). The development of BACE1 inhibitors has proven difficult for several reasons, one being that the enzyme binding site is very large, requiring a large molecule to inhibit it. Such a large molecule is not easily deliverable medicinally (De Strooper et al., 2010).

Gamma secretase was the last of the APP involved secretases to be discovered due to its composition complexity. Presenilin 1and 2 genes encode the structure for the main catalytic site while three other accessory proteins are also associated with the enzyme (Takasugi, 2003; Edbauer, 2003). This particular enzyme expresses a large

heterogeneity leading to a large array of functions depending on the different forms of presenilian genes making up the enzyme (De Strooper et al., 2010). APH-1B, one specific accessory protein has been shown to associate with gamma secretase and is shown to be involved with APP cleavage, (Serneels et al., 2009), but is also involved with Notch signaling and is lethal if knocked out. The removal of this particular protein inhibits APP cleavage but does not totally destroy the function of the enzyme, unlike when using an inhibitor of beta secretase. At this stage, there are several gamma secretase inhibitors on the market being used with some success, but targeting specific toxicity has proven to be difficult.

The approaches mentioned above are those that utilize antibodies that bind to the highly aggregated forms, but it is more beneficial to catch the amyloid beta before it reaches this form, therefore not allowing the degeneration to progress at all and allowing a patient to maintain better cognitive function. For this, there are antibodies that have been developed in order to bind specifically to the various regions on the amyloid beta molecule. There are antibodies developed to bind to the N-terminal, the mid region and the C-terminal regions (De Strooper et al.).

1.3 - Tau Hypothesis

The main protein component of the tangles is an abnormally phosphorylated from of tau. Tau was described as a microtubule associated protein, whose main biological function in vitro is to promote the assembly of tubulin into microtubules. Microtubules are one of the three components of the cytoskeleton and in an affected neuron from AD the microtubules are destroyed and they are replaced by tangles of Paired Helical

Filaments (PHF). Microtubules are like the train tracks that carry the axonal transport. There are proteins that stimulate tubulin assembly into microtubules and stabilize the preformed ones. They are known as Microtubule Associated Proteins (MAPs). In neurons, the main MAPs are MAP1, MAP2 and tau. Tau in the central nervous system is a family of six proteins derived from a single gene from the alternative splicing of the mRNA. It has an imperfect repeated sequence that binds to microtubules (microtubule binding domain, or repeats R). The human brain tau isoforms range from 352 to 441 amino acids. They differ in whether they contain three or four tubulin binding domains/repeats (R) of 31 or 32 amino acids each near the C-terminal and two, one, or no inserts of 29 amino acids each in the N-terminal portion of the molecule. The isoforms are developmentally regulated, in fetal brain the 3R isoform is the main one and the 4R taus are present in the adult brain. In AD, all six isoforms have been shown to be present in the PHFs in a hyperphosphorylated form. Filaments of hyperphosphorylated tau are not exclusive from AD. Other neurodegenerative diseases, such as fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), amyotrophic lateral sclerosis, cortical basal degeneration, dementia pugilistica, Pick's disease, progressive supranuclear palsy, and tangle-only dementia are characterized by deposits of hyperphosphorylated tau. This strong correlation of dementia with hyperphosphorylated tau became more relevant when in 1998 mutations on tau were identified as the cause of frontotemporal dementia. The presence of hyperphosphorylated tau precedes the appearance of tangles (Bancher). The pathological tau is modified and possibly gains a toxic function.

1.3.1 - Tau Biological Function

Tau is mainly found in neurons. Tau's main biological function is characterized *in vitro* is to promote the assembly of tubulin into microtubules. Different tau isoforms have different abilities to promote microtubule assembly. Tau is a phosphoprotein and the level of phosphorylation regulates its binding to microtubules. In AD tau is unable to bind to microtubules. Tau has been expressed in non-neuronal cells and the morphology of the cells changes. Tau makes bridges between microtubules, allowing them to form bundles. In a normal neuron, tau is concentrated in the axon. In culture, addition of tau antisense eliminates the formation of an axon, suggesting that tau is important in axon formation. Nevertheless, tau knockout mice were able to develop axons, though of smaller caliber, arguing that either the expression of tau is not important or because of its importance there are proteins that can replace tau's function. In a natural human knockout for tau, families with microdeletions of the tau gene result in a family with developmental problems.

Even though the most studied biological function of tau is related to tubulin assembly into microtubules, tau has been found associated with plasma membrane (Brandt et al., 1995) mitochondria (Rendon et al., 1990), and nucleic acids (Hua et al., 2003; Kampers et al., 1996), suggesting that it may act as a mediator between microtubules and these organelles. Binding of tau to the microtubules affects motility of motor proteins, such as dynein and kinesin (Dixit et al., 2008). The microtubule-binding domains of tau was sufficient to inhibit motor activity differentially for dynein and kinesin (Dixit et al., 2008), suggesting that tau could modulate axonal transport. Tau and other MAPs can interact directly or indirectly with the actin cytoskeleton (DiTella et al.,

1994; Fulga et al., 2007; Zmuda and Rivas, 2000). If tau is expressed at high levels in cultured cells result in cell toxicity and in the freezing of the mitochondria in the perinuclear region (Ebneth et al., 1998). Tau has also been seen in the nucleus of different cells, and recently, it has been postulated that tau-induced toxicity is related to the re-entry of neurons into the cell cycle. Tau is translocated into the nucleus in cells overexpressing tau, and this translocation induces apoptosis (Tsukane et al., 2007). In a recent study, it was claimed that tau over expression protects against apoptosis (Tsukane et al., 2007). We have previously shown that tau interacts with DNA (Hua et al., 2003). A recent study, in which the endogenous murine tau was knocked out and the human 4R tau was knocked in, showed that there were significant increases in hippocampal volume and neuronal number because of increased neurogenesis and neuronal survival (Sennvik et al., 2007). In the same study, the longest human tau isoform expression was found to suppress proliferation, promote neuronal differentiation, and restore neurite and axonal outgrowth.

In 2005 it was reported that tau expression in breast cancer cells was a marker for resistance to paclitaxel. The same correlation was found in prostate cancer and gastric cancer (Lee CAR in press). These studies suggested that tau could be participating in a different biological function independent of microtubules.

Also independent of microtubules in 2008 it was reported that tau interacts with histone deacetylase (Ding et al., 2008)and that the interaction inhibits the enzyme activity (Perez et al., 2009).

Tau biological function seems to be more complex than just a structural protein, we need to keep on working and learning on the biology of this interesting key protein.

1.3.2 - Tau Posttranslational Modifications

Besides the different tau isoforms generated by alternative splicing, tau can be posttranslationally modified in several ways, which include phosphorylation, glycosylation, ubiquitination, glycation, polyamination, nitration, and truncation. Conformational differences in tau molecule can arise from differences in the primary structure and/or from post-translational modifications. Post translational modifications are known to regulate tau's biological function.

1.3.3 - Tau Phosphorylation and protein kinases involved

Tau is a phosphoprotein with a physiological phosphorylation state of about 3 moles of phosphate per mole of protein, but in AD, this ratio is ~7–10 moles per mole (Köpke et al., 1993) and even possibly higher in tangles (Alonso et al., 2006; Ksiezak-Reding et al., 1992).

Hyperphosphorylation of tau is a pathological event and it means that new phosphorylation sites appear and the stoichiometry of phosphorylation increases. Hyperphosphorylation of tau precedes the appearance of tangles (Bancher et al., 1989; Iqbal et al., 1986; Köpke et al., 1993). There are at least 30 abnormal sites, which are mainly Ser or Thr, and 50% of them are canonical sites (Ser/Pro, Thr/Pro) for proline-

dependent protein kinases (PDPKs) (Hanger et al., 1998; Hanger et al., 2007; Morishima-Kawashima et al., 1995). The remaining sites are phosphorylated by non-PDPKs. Therefore, more than one kinase must be involved in the abnormal phosphorylation of tau.

Tau has been shown to be phosphorylated at Ser and Thr by several protein kinases, in vitro and in vivo, including GSK-3, cdk5, protein kinase A, CaM kinase II, protein kinase C, mitogen-activated protein kinase (MAP kinase), casein kinase I, and cyclin-dependent kinase 2 (cdc2) (Iqbal et al., 2005; Lee et al., 1998).

From all the kinases that have been shown to work on tau the one that received more attention is GSK3 kinase not only because it modifies several sites of the tau protein present in neurofibrillary tangles but also is able to modulate the generation of amyloid-beta, as well as to respond to this peptide. In several transgenic models, overexpression of GSK-3 has been associated with neuronal death, tau hyperphosphorylation and a decline in cognitive performance (Hernandez et al., 2010). Recently, GSK3 overexpression in a tau knockout resulted in a milder phenotype, suggesting that it is hyperphosphorylated tau mediates the pathology observed in the GSK3 transgenic mice (Gómez de Barreda et al., 2010).

Phosphorylation of tau decreases its interaction with microtubules, and certain sites such as Ser214, Thr231, Ser235, and Ser262 (numbering based on tau441) are the major sites for inhibition of the binding of tau to microtubules (Brandt et al., 1994; Drewes et al., 1995; Jenkins and Johnson, 1997; Sengupta et al., 2006; Sengupta et al., 1997; Singh et al., 1996). Furthermore, the phosphorylation of tau is influenced by prior

phosphorylation, e.g., cdk5 phosphorylation stimulates further phosphorylation by GSK-3β in vitro (Sengupta et al., 1997). Activation of PKA primes tau for hyperphosphorylation by the basal activity of GSK-3b and impairs spatial memory in normal adult rats (Liu et al., 2004c; Sun et al., 2005). Modulation of orthologues of GSK3beta and cdk5 in yeast transfected with human brain tau, resulted in conformationally modified tau that could self-assemble and induce in vitro wild-type tau aggregation (Vandebroek et al., 2006; Vandebroek et al., 2005). Tau phosphor-sites Thr212, Thr231 and Ser262 are present early in AD pathology (Alonso et al., 2004; Ksiezak-Reding et al., 1992; Liu et al., 2004a). Thr212 can be phosphorylated by the dual-specificity tyrosine(Y)-phosphorylation–regulated kinase 1A (Dyrk1A), coded in chromosome 21, and most individuals with trisomy 21 Down syndrome (DS) show early-onset of AD. The amyloid precursor protein (APP) is also coded in chromosome 21; therefore, with the extra copy of chromosome 21, DS patients have three copies of Dyrk1A and APP. Ryoo et al. (Ryoo et al., 2008) generated a transgenic mouse model overexpressing Dyrk1A. These mice have higher tau phosphorylation at Thr212, and this hyperphosphorylated tau did not promote microtubule assembly (Ryoo et al., 2008). Thr212 is in a very basic domain of tau, so the impact of phosphorylation on this site is very strong (Alonso et al., 2004).

Out of the five tyrosines 18, 29, 197, 310, and 394 (according to tau441), Tyrosines 18, 197, and 394 have been shown to be phosphorylated in the brain of patients with AD whereas tyrosine 394 is the only residue that has been described to date that is phosphorylated in physiological conditions. In 1998, tau was shown to be phosphorylated also at tyrosine by co-transfection with fyn in cell culture (Lee et al.,

1998). Subsequently, immunoreactivity against phospho-tyrosine was shown to be associated with tangles in AD brains (Lee et al., 2004).

Src family kinases and spleen tyrosine kinase (Syk) have been shown to phosphorylate tyrosine 18 while c-Abl is capable of phosphorylating tyrosine 394. AD and that Fyn and c-Abl are critical in the neurodegenerative process which occurs in tauopathies (Tremblay et al.).

In a recent report Azorsa et al, (2010) used a screen of 572 kinases in the human genome for effects on tau hyperphosphorylation using a loss of function, high-throughput RNAi approach. They found three kinases from this screen, the eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2), the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), and the A-kinase anchor protein 13 (AKAP13) on tau phosphorylation at the 12E8 epitope (serine 262/serine 356). They proposed that EIF2AK2 effects may result from effects on tau protein expression, whereas DYRK1A and AKAP13 are likely more specifically involved in tau phosphorylation pathways (Azorsa et al., 2010).

Another kinase implicated in tau phosphorylation is DNA damage-activated Checkpoint kinase 2 (Chk2) (Iijima-Ando et al., 2010). Overexpression of Drosophila Chk2 increased tau phosphorylation at Ser262 and enhanced tau-induced neurodegeneration in transgenic flies expressing human tau. The authors postulate that because there is accumulation of DNA damage in the brains of AD patients, it is concievable that the DNA damage-activated kinases Chk1 and Chk2 may be involved in tau phosphorylation and toxicity in the pathogenesis of AD.

1.3.4 - Tau Phosphorylation And Phosphatases Involved

The state of phosphorylation of tau is also controlled by phosphatase activities. PP-1, PP-2A, and PP-2B can dephosphorylate abnormally hyperphosphorylated tau in vitro (Gong et al., 1994; Wang et al., 1995). Although PP-2C can dephosphorylate tau that is in vitro phosphorylated with protein kinase A, it cannot dephosphorylate AD P-tau (Gong et al., 1994). PP-2A regulates tau phosphorylation directly, and indirectly by regulating GSK3 activity (Qian et al., 2010). PP5 can dephosphorylate tau at similar sites as PP-2A (Gong et al., 1994). Other phosphatases regulate tau phosphorylation indirectly, by regulating the activity of the kinases/phosphatases that act on tau, as in the case of the phosphatase and tensin homologue PTEN. It has been shown that the loss of PTEN in mouse cerebellar neurons causes neurodegeneration by hyperphosphorylation of tau and neurofilaments, and activation of Cdk5 and pERK1/2, suggesting that dysregulation of the PTEN/pAkt pathway can mediate neurodegeneration (Nayeem et al., 2007). PTEN has been shown to accumulate with tangles in neurons of patients with AD and that in those neurons PTEN changes its subcellular localization (Sonoda et al., 2010). The activities of kinases and phosphatases is regulated by different mechanisms: recently it has been shown in ovaroiectomized rats that progesterone regulates the phosphorylation of PP-2A and PTEN, affecting therefore tau phosphorylation (Amorim et al., 2010). The phosphorylation of tau is regulated also in the different sub cellular localization. Bertrand et al (2010) (Bertrand et al., 2010) studied tau phosphorylation in the somotodendritic compartment and in the axons and found that with phosphatase inhibitors epitopes on tau appeared phosphorylated, showing that in the axons tau is kept dephosphorylated by the activity of the phosphatases. (Bertrand et al., 2010).

In vitro tau can be dephosphorylated with overlapping specificities; however, their role in vivo is unclear. In human brain, PP2A and PP1 are the most abundant phosphatases, and PP2A has the major activity towards in vitro phosphorylated tau (Liu et al., 2005). PP-2A is a major tau phosphatase, and it interacts directly with tau (Gong et al., 2000; Sontag et al., 1999). Inhibition of PP-2A promotes tau hyperphosphorylation, microtubule destabilization, modification of synapse structure, and neurodegeneration (Bennecib et al., 2001; Gong et al., 1993; Merrick et al., 1997; Sontag et al., 1996). It was reported that the activity of PP-2A is significantly decreased in AD (Gong et al., 1993). Therefore, hyperphosphorylated tau in Alzheimer brain and possibly other tauopathies could be the result of a deficit in phosphatase activity, with a transiently elevated tau kinase activity or activities, because PP-2A regulates the activities of several tau kinases, including CaM kinase II (Bennecib et al., 2001), PKA (Li et al., 2004; Sengupta et al., 2006), and ERK1/2 (Kins et al., 2003; Pei et al., 2003).

1.3.5 - Tau Phosphorylation And Biological Activity

Tau is a phosphoprotein and the phosphorylation of tau regulates its binding to microtubules. In AD, tau is hyperphosphorylated. Hyperphosphorylated tau is present in neurons from AD patients as soluble specie and as pair helical/ straight filaments (PHF/SF) (Köpke et al., 1993). We have described an inhibitory function for hyperphosphorylated tau. Abnormally hyperphosphorylated tau from AD brains (AD P-tau) sequesters normal tau, MAP1, and MAP2 from the microtubules, and depolymerizes the microtubules in vitro (Alonso et al., 1994; Alonso et al., 1997). Microtubules support axoplasmic transport, and in the tangle-bearing neurons of patients with AD, the microtubule system is disrupted and replaced by PHFs. The microtubule dynamic appears

to be critical to the well-being of any cell, and tau regulates its dynamic both in vivo and in vitro in a neuron. Abnormally hyperphosphorylated tau from AD brain (AD P-tau) does not promote microtubule assembly (Alonso et al., 1994)and inhibits the assembly promoted by normal tau (Alonso et al., 1996; Alonso et al., 1994) and other MAPs (Alonso et al., 1997) in vitro and in extracted cells (Alonso et al., 2006; Li et al., 2007). AD P-tau binds normal tau and MAPs. This property makes the hyperphosphorylated tau an active molecule in disrupting the microtubule system. This property is lost upon dephosphorylation (Alonso et al., 2001b; Alonso et al., 1996; Alonso et al., 1997; Alonso et al., 2006; Alonso et al., 2004; Li et al., 2007). We have also shown that hyperphosphorylation of tau induces its self-assembly into filaments, and this property is also lost upon dephosphorylation (Alonso et al., 2001b). Nevertheless, filaments formed by hyperphosphorylated tau do not bind tau or disrupt microtubules, suggesting that the polymerized form of hyperphosphorylated tau is inert. Similar results were described when using a model of neurodegeneration: Drosophila expressing human tau on motor neurons the authors showed that soluble hyperphosphorylated tau was toxic by disrupting microtubules (Cowan et al., 2010).

A common approach used to study the relevance of tau phosphorylation sites is the use of pseudophosphorylation. A Caenorhabditis elegans model of tau hyperphosphorylation worms that are transgenic for tau pseudophosphorylated at several sites showed multiple dorsal and ventral discontinuities, with the dorsal cord appearing to be more severely affected. At all developmental stages, transgenic worms were more affected than the control line or wild-type tau transgenic worms. Discontinuities in the nerve cords are indicative of either incomplete neurite outgrowth during development or

a potential degeneration of neurites. The finding that the presence of pseudophosphorylated tau did not induce neuronal loss suggested that modified tau interfered with the intracellular mechanism of axonal growth and pathfinding (Brandt et al., 2009). Using Drosophila as a model, Steinhilb et al. (Steinhilb et al., 2007a) generated a transgenic fly expressing tau, with 14 phosphorylation sites mutated to Alanine avoiding phosphorylation. The neurotoxicity was abolished, suggesting that SP/TP phosphorylation of tau is required for the generation of abnormal conformations and for neurotoxicity (Steinhilb et al., 2007b). However, these authors showed that it is not a single site that is responsible for the toxic effect and that the presence of more than one site in concert is needed to exert tau-induced neurotoxicity (Steinhilb et al., 2007b). By screening 1250 mutant Drosophila lines, Blard et al. (Blard et al., 2007) identified several components of the cytoskeleton, and particularly from the actin network, as specific modifiers of tau V337M–induced neurodegeneration. This finding suggested that disruption of the microtubule network in presynaptic nerve terminals could constitute early events in the pathological process leading to synaptic dysfunction in tau V337M pathology.

1.3.6 - Other Posttranslational Modifications of Tau

It has been reported that AD tau is aberrantly N-glycosylated, and that this abnormal modification promotes tau hyperphosphorylation (Liu et al., 2002; Wang et al., 1996). Tau is also modified by O-linked monosaccharide b-N-acetylglucosamine (O-GlcNAc) through hydroxyl groups of serine and/or threonine residues. This is a novel

type of O-glycosylation (Arnold et al., 1996; Liu et al., 2004a; Torres and Hart, 1984), which is similar to phosphorylation and is highly dynamic with a reciprocal relationship between O-glycosylation and phosphorylation (Kamemura et al., 2002). O-GlcNAcylation negatively regulates tau phosphorylation and is down-regulated in AD brain (Liu et al., 2004a). PHF-tau has been shown to be poly- (Bancher et al., 1991; Iqbal and Grundke-Iqbal, 1991; Morishima and Ihara, 1994) and mono-ubiquitinated (Morishima-Kawashima et al., 1993). PHF-tau is also modified by glycation (for review, see Munch et al. (Münch et al., 2002)).

Although hyperphosphorylated tau is associated with all these diseases with tau deposits, other hypotheses have been proposed as a mechanism of tau-induced neurodegeneration. Truncation of tau has been observed both at Glu391 and Ser421 (Gamblin et al., 2003b; Novak et al., 1991). The truncated form of tau had been associated with apoptosis in cell culture cells (Fasulo et al., 2000). However, tau has been found as a whole molecule in PHFs in AD, and to date, there have been no quantitative data on the percentage of tau that is cleaved in AD.

In contrast to the abnormal hyperphosphorylation and glycosylation that are early events of tau pathology, glycation, like ubiquitination, is a late event. Glycation normally leads to subsequent oxidation, dehydration, condensation, and, finally, formation of heterogeneous products called advanced glycation end products (AGEs). It has been reported that tissue transglutaminase can incorporate polyamines into tau both in vitro and in situ (Tucholski et al., 1999). PHFs isolated from AD brain are immunoreactive to an antibody against tissue transglutaminase, suggesting that this enzyme may play a role in PHF formation (Dudek and Johnson, 1993; Miller and Johnson, 1995; Norlund et al.,

1999). Tau in AD brain has been shown to be nitrated (Horiguchi et al., 2003). In AD, tau is found as adducts of hydroxynonenal (HNE), and these modifications are controlled by phosphorylation (Moreira et al., 2005; Takeda et al., 2000)

1.3.7 - FTDP-17 Tau Mutations

Three different types of tau mutations linked to frontotemporal dementia (FTDP-17) have been described: missense mutations, intronic mutations, and a deletion mutation. The missense mutations result in the substitution of one amino acid. It has been reported that some of these mutations somehow compromise tau's ability to promote microtubule assembly (Hasegawa et al., 1998; Hong et al., 1998), and in the presence of polyanions, these mutations promote tau’s ability to polymerize into filaments (Gamblin et al., 2000; Goedert et al., 1999). The intronic 5´ to exon 10 mutations result in over-expression of four-repeat tau (4R taus) (Hutton et al., 1998; Spillantini et al., 1998). In the latter case, the argument that the mutations compromise tau’s ability to promote microtubule assembly cannot be made, because 4R tau is known to bind microtubules better than 3R tau does (Alonso et al., 2001b; Panda et al., 2003). The models proposing that mutant tau is more prone to self-assemble support a gain-of-toxic function. Other schools of thought propose an alternative loss-of-function model in which tau-mediated neuronal cell death is caused by the inability of affected cells to properly regulate their microtubule dynamic due to misregulation by tau. It has been shown that missense FTDP-17 mutations that alter amino acid residues near tau's microtubule-binding region modify the ability of tau to modulate microtubule dynamics (Feinstein and Wilson, 2005). A "microtubule misregulation" model has been proposed in which abnormal tau isoform expression

results in the inability to properly regulate microtubule dynamics, leading to neuronal death and dementia (Levy et al., 2005).

We have found that the FTDP-17 tau mutations R406W, V337M, G272V, and P301L make this protein a more favorable substrate in vitro for brain kinases (Alonso et al., 2004). With regards to FTDP-17 tau phosphorylation, the reports in the literature are quite variable. In cellular systems, it has been reported that mutant taus phosphorylated less than or equally as much as wild-type tau (DeTure et al., 2002; Ko et al., 2005; Mack et al., 2001; Matsumura et al., 1999; Pérez et al., 1996). The discrepancy with our report might be due to the absence of certain kinases in the cell lines used, to the different isoforms of tau transfected, and/or to the fact that tau can be microtubule-bound in the cell system. It has been shown that free tau is a preferred substrate over microtubule-bound tau for phosphorylation by several kinases (Sengupta et al., 2006). One of the most remarkable differences we have found in vitro with τ4L is the 12-times-faster phosphorylation at Ser396 of $\tau 4L_{R406W}$ and the more than 4-times-greater total phosphorylation at this site (Alonso et al., 2004). In cell lines, $\tau 4L_{R406W}$ seems not to be phosphorylated at Ser 396, although one study found it to be phosphorylated to a similar extent as wild-type tau in cell culture (Mack et al., 2001). When $\tau 4L_{R406W}$ is expressed in transgenic mice, it is found to be phosphorylated in vivo at several sites, including Ser396, and to be aggregated into filaments (Tatebayashi et al., 2002). When expressed in *Drosophila*, it is hyperphosphorylated but not aggregated into filaments (Wittmann et al., 2001). Expression of $\tau 4L_{G272V}$, $_{V337M}$, and $_{P301L}$ in transgenic mice showed increased tau phosphorylation and tau inclusions (Götz et al., 2001; Ho et al., 2001; Lambourne et al., 2005; Lewis et al., 2000; Tanemura et al., 2001). The exact molecular mechanism of

neurodegeneration in the affected patients is not yet understood. Like individuals with AD, individuals with the FTDP-17 mutation show accumulations of hyperphosphorylated tau as neurofibrillary tangles in every single mutation case. All the mutations discovered in tau are dominant, suggesting that the effect of tau mutations results in a gain- of-toxic function (Crowther and Goedert, 2000).

Our hypothesis is that the abnormal hyperphosphorylation of tau contributes to neurofibrillary degeneration in these clinically distinct diseases by disrupting the microtubule system, another form of "microtubule misregulation," and that tau phosphorylation can be modulated by its conformation.

1.3.8 - Self-Assembly of Tau

Unraveling the mechanism of self-assembly of tau has generated considerable interest. In vitro assembly of tau has been achieved under different conditions, such as urea treatment for 60 hours or incubations with unsaturated free fatty acids, tRNA, heparin, polyglutamic acid, or quinones; with a tau fragment; or with high protein concentration (12 mg/ml) and long incubation period (several days) (Crowther and Goedert, 2000; Goedert et al., 1996; Kampers et al., 1996; Montejo de Garcini et al., 1986; Santa-María et al., 2004; Wilson and Binder, 1995; Yanagawa et al., 1998). Quinones have been linked also to microtubule disruption, because of their interaction with tubulin (Santa-María et al., 2005). Nevertheless, in AD brain, tubulin does not appear to be the problem, because when this protein is isolated from Alzheimer brain, it has normal assembly properties (Iqbal et al., 1986; Khatoon et al., 1995). None of the conditions described above is compatible with the presence of the entire molecule, the six

isoforms of tau, or the hyperphosphorylated state. The levels of tau mRNA are not changed in Alzheimer brain compared to control brains (Mah et al., 1992). The levels of the tau protein in AD brains are increased to 7–10 times those of control brains, but this increase is more likely due to the hyperphosphorylation and deposit of the protein into tangles, making tau less prone to degradation (Khatoon et al., 1992). These are very important observations, because the levels of tau have to be dramatically increased in transgenic animals in order to detect any effect (Spittaels et al., 1999). Only transgenic mice expressing FTDP-17 mutated tau generate some phenotype with low levels of tau over-expression (Tatebayashi et al., 2002). Taken together, these observations suggest that in AD brain, it is not the level of tau, but the appearance of a modified form of tau—of a conformationally different molecule—that makes a difference. It has also been proposed that a conformational change of tau molecule precedes its aggregation into PHFs and is one of the earliest events in AD (Weaver et al., 2000). This conformational change in tau molecule can be seen with the use of the antibodies Alz50 and MC1 (Jicha et al., 1997). The conformation required for tau to be recognized by these antibodies can also be acquired by hyperphosphorylation. We have also shown that AD P-tau polymerizes readily into tangles of PHF/SF, that dephosphorylation abolishes tau's self-assembly, and that hyperphosphorylation of recombinant tau by brain kinases induces its self-assembly into tangles of PHF/SF (Alonso et al., 2001b; Wang et al., 2007). These results taken together suggest that tau's self-assembly is regulated by phosphorylation.

We have used several tau constructs to induce self-assembly and have found that tau's microtubule-binding domain polymerizes into filaments. Different groups have shown that the stretch of tau involved in tau-tau interaction lies within the microtubule-

binding domain (Crowther et al., 1992; Gamblin et al., 2003a; Gamblin et al., 2003b; Mukrasch et al., 2005; Pérez et al., 2001; Pérez et al., 1996). On the basis of our studies of self-assembly, we have postulated that both flanking regions of the microtubule-binding domain of tau inhibit its self-assembly: when we used peptides with the microtubule-binding domain, they polymerized readily, whereas tau constructs that included microtubule-binding domain and the N-terminal portion of tau and/or the C-terminal end of the protein abolished the self-assembly (Alonso et al., 2001b). We also postulated that the positive charge of tau molecule, concentrated in patches in the flanking region of tau, could be responsible for the inhibition of the binding, since the presence of the two N-terminal inserts of tau, which are highly negative, induced tau self-assembly (Alonso et al., 2001b). Upon phosphorylation, tau also acquires the ability to bind normal tau. Tau acquires the maximal ability to bind normal tau after the incorporation of about 4 moles of phosphate per mole of protein (Alonso et al., 2004), and it polymerizes after 10 moles of phosphate per mole of protein (Alonso et al., 2001b; Alonso et al., 2004). These results suggest that at least two different conformational states of tau are induced by phosphorylation: one in which the hyperphosphorylated tau is able to bind normal tau, and one in which it is able to self-assemble into filaments. These results, combined with the data of phosphorylation kinetics (see Alonso et al. (Alonso et al., 2004)), suggest that the conformation of tau needed to sequester normal tau might involve phosphorylation of tau at positions 199, 202, 205, 212, 235, 262, and 404, and for self-assembly, further phosphorylation at positions 231, 396, and 422. Phosphorylation at Thr 181 or Thr 217, sites with slower kinetics, might control the assembly, or these sites might be phosphorylated on the polymer. As evidence that a conformational change is

induced by FTDP-17 mutations, these mutated taus not only are better substrates for brain kinases but also polymerize into filaments at a lower stoichiometry of phosphorylation than the wild-type protein (Alonso et al., 2004).

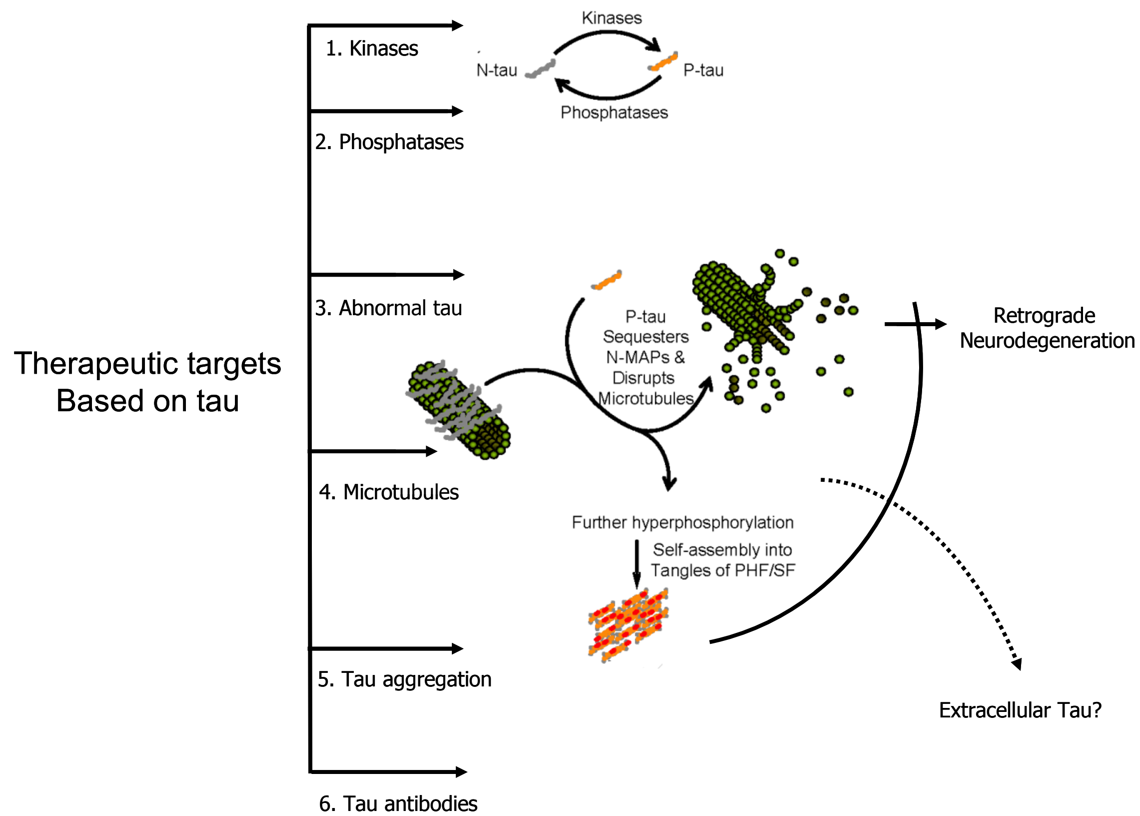
Tau has been shown previously to undergo intermolecular association through the microtubule- binding domains, and the flanking regions appear to be inhibitory of tau self-assembly (Alonso et al., 2004; Pérez et al., 2001; von Bergen et al., 2000). If we consider that tau has little secondary structure and that segments of tau are separated by prolines that induce a bend in the amino acid chain, the theoretical isoelectric points of these segments have a strongly basic charge at the N-terminal region to the microtubule-binding domains. We propose that these very basic segments (pI>9) in the proline-rich region of tau mask the intermolecular attraction of the microtubule-binding domains. Considering that the mutant taus start polymerizing after the incorporation of 4–6 moles of phosphate per mole of protein, the sites that are phosphorylated at a level of about 50% of the total phosphorylation of t4L in less than 2 h are Thr212, Ser235, and Ser262 in the N-terminal side. The pI value for the segments containing Thr212 is 12; Ser 235, 9.18; and Ser262. Phosphorylation of these sites decreases the theoretical pI, and consequently, the negative effect of the N-terminal region on tau self-assembly. The C-terminal region is basic up to Pro 397, and the rest of the segments are acidic. It is possible that the acidic segment “masks” the interacting part of tau and that the phosphorylation at Ser396 and/or Ser404 opens up this segment, allowing the intermolecular interaction through the microtubule-binding domains. Recently, it has been shown that phosphorylation at Ser262 is critical for abeta induced toxicity (Iijima-Ando et al., 2010)

Several ways can induce tau polymerization with similar outcomes. It is conceivable that given the variety of the tauopathies, more than one mechanism is involved in the disease. Nevertheless, up to now, there is no known human disorder with accumulation of tau that is not hyperphosphorylated.

1.3.9 - What Is Inhibitory—The Cytosolic Or the Polymerized Abnormally Hyperphosphorylated Tau?

It is conceivable that the mechanism of tau-induced neurodegeneration involves microtubule disruption. Recently, another putative link has emerged from the experiments in Drosophila. Khurana et al. (Khurana and Feany, 2007; Khurana et al., 2006) have described the cell cycle reentry as a downstream event of tau phosphorylation and in the case of neuronal cells. This reentry attempt is followed by apoptosis. This neurotoxicity is independent of tau aggregation and is exacerbated by oxidative stress (Dias-Santagata et al., 2007). We have found that pseudophosphorylated tau translocates into the nucleus (Alonso et al., 2010). Recently, using a Drosophila expressing hyperphosphorylated tau they were able to show that this soluble hyperphosphorylated protein was able to disrupt microtubules and compromise normal tau in vivo (Cowan et al., 2010), as we have previously proposed based on our biochemical studies

Figure1.1



1.4 - TAU THERAPEUTIC TARGETS

The lack of phenotype on the tau knockout mice (Harada et al., 1994; Ikegami et al., 2000; Takei et al., 2000) and the findings that AD P-tau inhibits and disrupts microtubule assembly it appears that hyperphosphorylated form of tau has gain toxic function and this is more toxic than the lack of tau function. Based on the tau hypothesis, due to a phosphorylation/dephosphorylation imbalance tau gets hyperphosphorylated, detaches from microtubules, sequesters normal tau and other MAPs, self-assemble into tangles of pair helical filaments and the neurons lose the microtubular network and undergoes retrograde neurodegeneration. This proposed mechanism, described in Fig 1, indicates several levels of therapeutic interventions:

1.4.1 - Kinases

As discussed before, more than one kinase has to be activated in order to generate abnormally hyperphosphorylated tau. Problems in selecting an appropriate kinase as a target arise from the number of sites that have been found to be phosphorylated in AD P-tau. We have described in the section of tau phosphorylation this complex process and the possible functional implications on tau biology. It is not really known which of tau phosphorylation sites are important on tau, what is the kinetics of phosphorylation in vivo, which is just a product of being sitting on a polymer, unable to be degraded. We have recently published a study showing that the combination of phosphorylation on tau at Thr212, Thr231 and Ser262 makes tau acquired a toxic behavior (Alonso et al., 2010). As we described before, it has been shown using Drosophila that several phosphorylation sites must work in concert (Steinhilb et al., 2007a). Therefore, it results complicated to

pick one kinase activity as a target for Alzheimer disease. As we describe in the previous sections, from all the kinases that have been shown to work on tau the one that received more attention is GSK3 kinase not only because it modifies several sites of the tau protein present in neurofibrillary tangles but also is able to modulate the generation of amyloid-beta, as well as to respond to this peptide. Dysregulation of GSK-3 activity is believed to play a key role in the pathogenesis of CNS chronic disorders such as Alzheimer's disease (AD), bipolar disorder, and Huntington's disease, and of metabolic disorders such as type II diabetes. In several transgenic models, overexpression of GSK-3 has been associated with neuronal death, tau hyperphosphorylation and a decline in cognitive performance (Hernandez et al., 2010). Recently, GSK3 overexpression in a tau knockout resulted in a milder phenotype, suggesting that it is hyperphosphorylated tau mediates the pathology observed in the GSK3 transgenic mice (Gómez de Barreda et al., 2010). In 2006, Engel et al. (Engel et al., 2006) describe the complete reversal of the tau-induced phenotype in a conditional mouse model of GSK 3 activity. All these evidence taken together, besides that the inhibition of GSK3b is well tolerated, made GSK3b a great therapeutic target. Different compounds, from cations like Li to small chemical compounds developed by different companies and even interference RNA have been used to decrease the activity of GSK3b (recently reviewed in Hernandez et al., 2009 (Hernández et al., 2009)). Other beneficial effects of GSK3b inhibitors is that it has been shown that the oral administration of a GSK3 inhibitor to adult wild-type mice or to amyloid precursor protein/presenilin 1 mice modeling Alzheimer amyloidosis significantly increased brain IGF-I content (Bolós et al., 2010). Reduced brain input of serum insulin-like growth factor I (IGF-I), a potent neurotrophic peptide, may be

associated with neurodegenerative processes, therefore making the effect of inhibiting GSK3 very desirable. Moreover GSK3 activity is also related to inflammation processes. Inhibition of GSK3 activity in animal models downregulates inflammation and the effect of inhibiting this activity might be beneficial in more than one way (reviewed by Jope et al, 2007 (Jope et al., 2007)). Since GSK3 participates in several processes, manipulation of this activity has to be done very carefully, since it is also involved in glucose metabolism and cancer, but to control the activity of this enzyme is very challenging and several pharmaceutical companies are working towards this aim.

Cdk-5 is another candidate kinase that phosphorylates tau and could participate in generating the abnormal conformation of tau. Cdk5 inhibitors, such as roscovitine, has been used in the triple transgenic animal model and it has been shown decreased levels of phosphorylated tau (Kitazawa et al., 2005). When tau was overexpressed in yeast and the orthologue of cdk-5 was inhibited tau was very much hyperphosphorylated (Vandebroek et al., 2005) probably because the activity of cdk5 regulates that of GSK3: inhibition of cdk5 led to activation of GSK3 (Wen et al., 2008).

Other kinases are putative kinases for tau and their activities could be target to inhibit tau-induced neurodegeneration, may be the most effective approach would be to target combination of kinases, such as the one proposed by the University of Illinois at Chicago, to inhibit GSK3 and CK2. Another recent study showed that the use of a small inhibitor that was able to target GSK-3β, ERK2/cdc2, PKA, and PKC reducing tau phosphorylation in a transgenic mouse model (Le Corre et al., 2006). Kinase inhibition, mainly GSK3b, is an approach that we are eagerly waiting for the results of the trials.

1.4.2 - Phosphatases

Since tau hyperphosphorylation could be a result of high kinase activity or decreased phosphatase activity, to inhibit kinases or to activate phosphatases are attractive therapeutic targets. PP2A is the main tau phosphatase in brain; and the activity has been shown decreased in AD brains (Gong et al., 1993) therefore it has been the center of attraction for drug development. Nevertheless, unlike kinases, phosphatases have a wider range of substrates and it makes them more prone to give undesirable secondary effects.

PP2A activity is tightly regulated in the cells, two polypeptides have been identified as PP2A inhibitors: I_1PP2A and I_2PP2A (Li et al., 1996a; Li et al., 1996b).

In Alzheimer disease (AD) brain, the level of I_1 PP2A, is increased, the activity of the phosphatase is decreased, and tau is abnormally hyperphosphorylated. The mechanism of inhibition is starting to be elucidated. Targeting these inhibitors is another approach to prevent hyperphosphorylation of tau.

1.4.3 - Abnormal tau

Tau from Alzheimer disease brain does not behave biologically as normal tau. Certain conformational antibodies recognieze a different conformation of tau. Several neurodegenerative diseases are characterized by the presence of misfolded proteins. The cellular alternatives will be to promote degradation of the proteins. Hsp70/CHIP chaperone system plays an important role in the regulation of tau turnover and the

selective elimination of abnormal tau species. Hsp70/CHIP may therefore play an important role in the pathogenesis of tauopathies and also represents a potential therapeutic target (Petrucelli et al., 2004). The heat shock proteins response, which can mediate the degradation of misfolded proteins, can be modulated pharmacologically and this has been recently reviewed (Deture et al., 2010).

1.4.4 - Microtubules

In Alzheimer disease and related neurodegenerative diseases the microtubule system is destroyed, compromising the stability of the synapses. The more we know about the disease, the more we believe that cytoskeletal disruption and synaptic impairment are early events in the process of neurodegeneration. Hyperphosphorylated tau appears early in the disease progression and this modified form of tau inhibits microtubule assembly and self-assembles into PHF (Alonso et al., 2001b; Alonso et al., 1996). Given this scenario, it is very attractive to study the effects of different components able to stabilize the microtubule system, to compensate for the early disruption of the cytoskeleton. The idea of using drugs that stabilizes the microtubule structure appears attractive and promising (Trojanowski et al., 2005). Dr. Gozes and collaborators proposed to use peptides, derived from the Activity-Dependent Neuroprotective Protein (ADNP) and tau mimetic to study its effect on microtubule stability, its ability to bind to tubulin and MAPs, as well as promoting cell survival (Shiryaev et al., 2009).

The use of peptides in Alzheimer therapeutic efforts is growing and during the course of these studies, several peptides have shown positive neuroprotective effects on

amyloid beta polymerization, toxicity, secretases activity. Humanin is another peptide that has been studied and shown to induce neuroprotection in several different insults present in Alzheimer diseased brain (Xu et al., 2010; Zhang et al., 2009). Dr. Gozes and collaborators attacks the problem from the perspective of the cytoskeleton. They used picomolar concentration of the peptides (from NAP to Al-108) promotes cell survival, by interacting with tubulin and stabilizing the microtubules (reviewed by Gozes et al., 2009 (Gozes et al., 2009)). Using NAP at 1pM concentrations, in a polymerization system containing tubulin and tau, they found that tau was increased twofold in the microtubule fraction (Gozes and Divinski, 2007). They analyzed these results by electron microscopy and found that the density of the microtubules was also increased. Surprisingly, NAP induced a selective selection of tubulin isoforms: where the microtubules density was increasing, the content of βIII tubulin (neuron specific tubulin isoform) decreased, suggesting that the presence of NAP at that low concentration could be modulating the dynamics of the microtubules by modifying the isoform composition of the microtubules. Battacharya and Cabral (2004) (Bhattacharya and Cabral, 2004) reported that the expression of V β tubulin disrupts the microtubule and that this effect is reversed with the addition of taxol, supporting the idea that a selection of tubulin isoforms can modify the dynamics of the microtubules.

In tauopathies, such as AD, one of the first steps in neurodegeneration is that the microtubules system generates retrograde neurodegeneration leading to a loss of synapses. The therapeutic approaches most likely to be successful should target the microtubule stability, either by controlling tau phosphorylation levels or interacting with the microtubule protein to stabilize microtubule structure.

1.4.5 - Tau aggregation

The presence of hyperphosphorylated tau that disrupts microtubules constitutes a threat to the stability of the neurons. Microtubules support axoplasmic transport, and in the tangle-bearing neurons of individuals with AD, the microtubule system is disrupted and replaced by PHF. Microtubules are polymers of tubulin, and it is well known that the degree of tubulin polymerization in a cell has critical consequences on the fate of the cell. Agents that either stabilize or disrupt microtubules can induce apoptosis in many cell types, especially in the neurons, in which the processes are long and the structure and transport are supported by microtubules. Whether tau is a toxic entity for the cells in its polymerized form is a subject of debate. SantaCruz et al. (Santacruz et al., 2005), using transgenic mice expressing inducible human four-repeat tau with the P301L mutation, found that the cognitive deficiencies correlate with the appearance of soluble hyperphosphorylated tau. When tau expression was turned off, there was no clearance of the polymerized tau, soluble tau decreased, and cognition improved, suggesting that the polymerized tau was not sufficient to cause cognitive decline or neuronal cell death. Andorfer et al. showed that in human tau transgenic mice, while there was widespread neurodegeneration, the PHF-containing neurons appeared "healthy" in terms of nuclear morphology, suggesting that the polymerized protein was probably neuroprotective (Andorfer et al., 2005). Previously, a similar conclusion was inferred from a morphometric study of brain biopsy specimens from AD and control cases, which found that the appearance of tangles does not necessarily herald the demise of a neuron in AD (Bondareff et al., 1989). Another morphometric study showed that the decrease in microtubule density was unrelated to PHF accumulation (Cash et al., 2003). It has been

demonstrated that the formation of inclusion bodies, in the case of Huntington disease, reduces the level of mutant huntington and the risk of neuronal death (Arrasate et al., 2004). We have shown that polymerization of hyperphosphorylated tau into filaments, i.e., PHF/tangles, makes it inert, which unlike the non-polymerized AD P-tau, does not bind normal tau or tubulin and inhibit the microtubule assembly (Alonso et al., 2006; Iqbal et al., 1994; Khatoon et al., 1995). These findings suggest that inhibition of the hyperphosphorylation of tau rather than of its polymerization into PHF/tangles might hold therapeutic promise for AD and related tauopathies.

An inverse relationship has been found between the number of extracellular tangles and the number of surviving neurons (Cras et al., 1995). Thus, these data would suggest that the neurons that degenerate developed tau aggregates 3. We have proposed that hyperphosphorylated tau sequesters normal tau, disrupts the microtubule system, and self-assembles into tangles of filaments, a mechanism of tau toxicity in which mainly the cytosolic form of hyperphosphorylated tau disrupts the microtubule network, whereas on polymerization into PHFs, this inhibitory activity is lost (Alonso et al., 2006). PHFs accumulated in the cell body, though initially neuroprotective, might become deleterious when this space-occupying lesion in the neuron compromises its normal function, justifying the inverse correlation of extracellular tangles with neuronal survival. More recently, Khlistnova et al. (Khlistunova et al., 2006) showed that aggregates of a fragment of tau in cells are toxic, and inhibition of the aggregation decreased toxicity. Nevertheless, this last study was done with only a fragment of tau, which can behave completely differently from the whole molecule used in the previous studies. Another consideration will be to consider degree of tau aggregation, not in the form of tangles, but

as oligomers, and they propose that the inhibition of the oligomerization of ttau holds a therapeutic promise (reviewed by Takashima, 2010 (Takashima, 2010)).

1.4.6 - Tau Antibodies

It has been proposed that tau can be released to the extracellular space and that it could exert its toxic effect on other neurons. It was also proposed that tau can transfer the disease. Tau was used in two tangle mouse models to induce active immunization and it indicates that active immunization targeting an AD phospho-tau epitope reduces aggregated tau in the brain and prevents/slows progression of the tangle-related behavioral phenotype, including cognitive impairment (Boimel et al., 2010; Sigurdsson, 2009). These antibodies enter the brain and bind to pathological tau within neurons although the therapeutic effect may at least in part be due to clearance of extracellular tau that may have biological effects.

1.5 - CONCLUDING REMARKS

The protein component of the plaques was identified in 1984 as beta amyloid (Glenner, G.G., Wong, C. W. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. 120: 885-890). The protein component of the tangles was identified two years latter, in 1986 as tau, the neuron-specific microtubule associated protein (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b). Tau normally binds to and assists in microtubule formation; faulty microtubule formation could have dramatic effects on neurons. Tau is altered in a whole set of neurodegenerative diseases including AD. The

stability of the cytoskeleton, known to be greatly affected in AD, impairs the neuronal transport and connectivity. The neurofibrillary degeneration correlates with the progression of the disease. A two year lag in describing tau (coupled with a lack of genetic association with tau) has led to retardation in tau research, leaving tau research well behind that of Aβ. Thus, research on Aβ leads the field of Alzheimer's disease (AD). What has been gleaned from the nearly two decade research in AD is that the disease is a multifactorial disease and we are far from been able to prevent the disease. The trials towards lessening Aβ pathology have shown that the patients do not benefit by treating the plaques deposits in the mild to moderate stages of the disease. The clinical results raise questions as to the validity of Aβ as the principle cause of the AD pathology. Couple these findings with the ubiquitous presence of tau fibrillary tangles in several dementias suggests that a focus to inhibit neurofibrillary pathology as well is needed to achieve clinical improvement. We foresee that any therapy in order to be successful should target the process of neurofibrillary degeneration.

CHAPTER TWO

Hyperphosphorylated Tau-induced cellular Pathologies; more than microtubule disruption

Abstract: Hyperphosphorylation of the microtubule associated protein tau is involved in several dementias that classify as tauopathies. In these diseases, tau is known to bind to itself rather than associate with microtubules. Without cytoskeletal support, neurons begin to loose their ability to function and therefore there is a loss of neuronal arborization. Earlier work investigated specific phosphorylation sites, while this work looks at certain specific sites using live cell imaging to better understand how the degenerative process is taking place. When cultured CHO cells express wild type tau, the microtubule network is healthy and shows normal microtubule movement and tau only associated with the microtubules. When expressing pathological human tau (PH-tau, pseudophosphorylated at T212, T231, S262) in CHO cells in culture, however, the cells have tau present throughout the cytoplasm, rather than associated with microtubules. A healthy microtubule network is not distinguishable in PH-tau expressing cells. Additionally, the cells experience membrane zeiosis, or excessive membrane blebbing, which are released into the surrounding environment. The zeiosis can be a cellular mechanism to remove toxic substances from the cell. The blebbing of the membrane causes the cells transfected with PH-tau shrink in size. Internally the presence of excessive cytoplasmic vacuoles are detected as well as aggregated protein in the form of filaments. Additionally, PH-tau is translocated into the nucleus. The exposure of wild type expressing cells to okadaic acid shows the appearance of membrane blebbing, lack of microtubule structure and translocation into the nucleus. Additionally, all three sites used in the PH-tau construct are phosphorylated when wild type tau is exposed to okadaic acid. Taken together, these results suggest that PH-tau does play a role in neurodegenerative process and that this process extends further than just microtubule disruption.

Keywords: tau, tauopathies, Alzheimer's disease, zeiosis, neurodegeneration

2.1 - INTRODUCTION

Tau is a microtubule-associated protein (MAP) that is specific to the axons of neuronal cells. Microtubules are assembled in the initial segment of the axon, and are responsible for both structural support and axonal transport. Most of the components required by the axon are synthesized and packaged in the cell soma. These components consist of neurotransmitters as well as mitochondria to provide energy to propagate signal transmission. The stabilized microtubule network is the "highway" by which all of these molecules are moved from the soma to the axon terminal via the activity of the motor proteins (dyanin and kinesin) (Cho and Vale, 2012; Hirokawa, 2011). This cellular trafficking is vital to the continuous function of the neurons. Without this transport, the neuron will no longer transmit signals and this loss of activity will lead to neurite retraction.

There are six tau isoforms seen in the human brain. These isoforms are generated by alternative splicing of the pre-mRNA. These can include three or four microtubule binding domains, all which reside at the C-terminal of the protein as well as zero, one or two 29 amino acid inserts at the proteins' N-terminal (Goedert et al., 1989; Himmler et al., 1989). Proper folding of these isoforms is critical for normal tau function.

The dysfunction of tau protein leads to a family of diseases called tauopathies, which include both Pick's disease, Alzheimer's disease and Fronto-temporal dementia with Parkinsonsim linked to chromosome 17. In these diseases, tau no longer binds to microtubules, but rather binds to itself and forms both paired helical and straight filaments (Goedert et al., 1996; Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Iqbal et al., 1986). Together these filaments aggregate to form the neurofibrillary tangles.

In Alzheimer's disease, these filaments inside the cell as well as the extracellular amyloid beta plaques that are the characteristic lesions used to diagnose the disorder after death. In its pathological state, Tau no longer performs the function of microtubule stabilization, but rather aggregates with itself (Alonso et al., 2001b; Alonso et al., 2008; Gozes et al., 2009). Without the binding of tau to the microtubules, the axonal network begins to fall apart. With the loss of the stabilized microtubules, there is now no further axonal transport, which leads to compromised neuronal signaling and leads to a retraction of neurites, which is the main cause of reduced brain matter in these patients.

So, the question as to how tau becomes pathological arises. There are several post translational modifications that occur on tau, which include acetylation (Irwin et al., 2012), glycosylation (Gong et al., 2005; Liu et al., 2004a; Smet-Nocca et al., 2011) and phosphorylation (Grundke-Iqbal et al., 1986c; Iqbal et al., 2009). All of these known post-translational modifications seem to contribute to tau pathology, but the best studied and most notable is phosphorylation. At normal levels there is approximately 3 moles of phosphate per molecule of protein. In a hyperphosphorylated state, the protein carries around 8 moles of phosphate per molecule of protein (Köpke et al., 1993). When in its hyperphosphorylated form, there are additional amino acid residues that become phosphorylated that are not normally phosphorylated in healthy functioning tau protein. The sites that are most commonly seen phosphorylated on hyperphosphorylated tau are Thr212, Ser214, Thr231, Ser235, and Ser262 (Brandt et al., 1994; Drewes et al., 1995; Jenkins and Johnson, 1997; Sengupta et al., 1997; Singh et al., 1996). These sites are also shown to play a major role in tau self assembly (Alonso et al., 2004).

Previous work has shown that *in vitro* that hyperphosphorylated tau induced self-assembly and the formation of filaments as well as the inability to promote microtubule assembly (Alonso et al., 2008; Alonso et al., 2001c). It has also been shown that phosphorylation at Ser262 accelerates tau assembly into filaments.

Additionally, the discovery of mutations on the tau gene have demonstrated that mutations of the tau gene alone are enough to promote cell death and subsequently dementia (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). There are three reported types of tau mutations; missense (Chang et al., 2008), deletion (Kovacech and Novak, 2010) and intronic (Wang et al., 2010a). Patients with mutations on chromosome 17 associated with Frontal-temporal Dementia have displayed neurons containing neurofibrillary tangles and cell death suggesting the pivotal role of this residue in tauopathies. Previous work has shown that *in vitro* the R406W mutation has given Tau the ability to self-assemble when containing approximately half the level of phosphate molecules when compared to wild type Tau (Alonso et al., 2004). When tau is hyperphosphorylated, it is hypothesized that a conformational change that takes place that makes the protein no longer able to bind to microtubules and that this toxic state is more easily reached if the protein carries the R406W mutation (Alonso et al., 2008).

Using a pseudophosphorylation at T212, T231 and S262, we have shown that tau no longer binds to microtubules and induces apoptosis in up to 85% of transfected cells (Alonso, et al., 2010). The addition of the R406W inherited mutation on tau enhanced the level of apoptosis (Alonso et al., 2010). No single phosphorylation or point mutation inhibited tau’s ability to bind to microtubules (Alonso et al., 2010). Tau’s inability to bind to microtubules only occurred when multiple sites were pseudophosphorylated.

Phosphorylation at S262 seemed to enhance the effect. This data supports the finding that specific sites of phosphorylation, when together, drive the protein to become toxic, rather than just the quantity of phosphate associated with the protein.

To further understand the mechanism of neurodegeneration, the pseudophosphorylation constructs from our previous studies were used in a live cell imaging paradigm. Initially, live imaging showed that all of the PH-tau transfected cells exhibited an excessive amount of membrane blebbing (zeiosis). This is a normal cellular occurrence when the membrane bulges out and is then reabsorbed back into the cell or released into the area to remove cellular waste. Zeiosis is a cellular process where the blebs are not ever reabsorbed by the cell, but rather are let loose into the surrounding area (Rose, 1966).

Membrane integrity has great implications to overall cell health, but little work as been explored on membrane damage in neurodegenerative diseases. It has been shown that blunt spinal cord injury leads to membrane disruption and that this level of disruption has been correlated to the severity of the traumatic injury (Simon et al., 2009). However, it has been shown that 3-morpholinosydnonimine (SIN-1) induced apoptosis leads to lipid peroxidation and membrane blebbing in cell culture (Ebadi et al., 2005). In relation to tau hyperphosphorylation, SIN-1 has been implicated in the overexpression of glycogen synthase kinase-3beta (GSK-3beta) and the aggregation of tau in rats that were injected with SIN-1 (Zhang et al., 2006). Taken together, membrane instability plays a major role in neuron functionality.

We turned to live cell imaging techniques to describe in real-time the occurrence of zeiosis in PH-tau expressing CHO cells. We also presented here novel quantification methods for describing blebbing in these cells.

2.2 - MATERIALS & METHODS

2.2.1 - Generation of GFP fused Pseudophosphorylated Tau Constructs & Plasmid Purification

The pseudophsohprylated tau vectors were generated by site directed mutagenesis according to Alonso et al. (2010) (Figure 2.1). The original pEGFP vector containing wild type tau was a gift from Dr. Michael Novak (Institute of Neuroimmunology, Bratislava, Slovak Republic). The GFP-fused pseudophsohprylated tau constructs were prepared specifically for this work in order to visualize tau in a live cell imaging system. To do this, the pAcGFP-C1 vector (Clontech) was digested with BglII and EcoRI (Promega). The pseudophsphorylated tau sequence was restricted with the same enzymes from the original, non-fluorescent vectors used in the previous work. These particular enzymes allowed us to insert tau in reading frame with the GFP gene and create a fusion protein. The pAcGFP vector was ligated with the tau gene using T4 ligase according to the manufacturer (Promega). The ligation mixture was then heat shock transformed into chemically competent *E.coli* (Fisher Scientific) and plated on Lauria Bertani plates (LB; 10g NaCl, 10g tryptone, 5g yeast extract in distilled water, autoclaved at 121°C at 15 psi for 15 minutes) which were supplemented with kanamycin (0.1mg/ml, from Fisher Scientific). Colonies were screened for the tau gene by restriction digest of purified plasmid and those cells positive glycerol stocked at -80°C. To purify plasmid DNA,

freezer stocks were re-plated in LB plates supplemented with kanamycin, incubated overnight at 37°C and a single colony was re-suspended in LB broth supplemented with kanamycin. Plasmid were isolated using the Promega Wizard Plus SV DNA purification kit (Promega) according to the manufacturers protocol.

2.2.2 - Cell lines used and transfection

Chinese hamster ovarian fibroblast cells (CHO) were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS, from Fisher Scientific) and 100 units of penicillin, 100μg streptomycin (Invitrogen, as a 100x solution). Cells were grown at 37°C in the presence of 5% CO_2. The day before transfection, cells were plated to 70-80% confluency. The plating vessel was determined by the type of experiment and will be mentioned in each section.

Cells were transfected with the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. Generally, cells were maintained for 48 hours prior to imaging or fixation.

2.2.3 - Live cell imaging

Cells were plated on Sykes Moore coverslips (25mm No. 2, Bellco Glass) in 10 cm^2 petri dishes. The cells were transfected according to the procedure above. Fourty eight hours post transfection, the cover slips were placed into the Sykes-Moore Chamber (Bellco Glass) using the 2.5mm gasket. This live cell imaging chamber allowed for the coverslip to be sealed into a closed system and filled with media. If kept at 37°C, the cells would survive for several hours on the microscope stage. The stage was warmed using a

ASI 400 Air Stream Incubator external stage warmer (Nevtek) and the temperature was monitored using a strip thermometer placed on the surface of the microscope stage. The samples were imaged using a Nikon C1 confocal laser scanning microscope. The images were captured using no frame averaging and the time interval was one frame captured every 3.5 seconds over a period of up to five minutes. This short time interval allowed for the capture of microtubule dynamic events and the movement of intracellular organelles. Movies were opened and analyzed using ImageJ with an ICS/IDS plugin.

2.2.4 - Cell Size Determination

In order to determine the size of the cells, light microscopy images were taken using a Zeiss Axioskop epi-fluorescent microscope equipped with a Zeiss MRc5 digital camera. Images were taken using the 20x objective, enough so that the cell periphery could be easily seen, but there are also many cells in the field of view. The perimeter of the cells was measured using ImageJ. The results were collected, analyzed the perimeter measurements were graphed using Microsoft Excel.

2.2.5 - Immunocytochemistry

Fixed cells were washed with PBS, permeabilized with 0.1% Triton X-100 (Fisher Scientific Cat # BP151-100), 0.1M glycine (Fisher Scientific) treatment to quench aldehyde autoflorescence, blocked in 4% donkey serum in PBS with 0.01% tween 20 (PBST) and incubated overnight in primary antibody. The following day, the cells were washed with PBS and incubated in PBST with secondary antibody for one hour, washed again with PBS and coverslipped with Vectorshield antifade mounting medium (Vector

Laboratories Cat #H1000). Cells were imaged using a Leica SP2 AOBS confocal laser scanning microscope. Images were analyzed using ImageJ.

2.2.6 - Okadaic Acid treatment

CHO cells were plated in eight well chamber slides (Lab-Tek II) and transfected the following day with fluorescent wild type Tau (pAcGFP-wtTau). Twenty-four hours after transfection, the cells were treated with either 100nM okadaic acid mixed in complete growth medium (Sigma-Aldrich Cat #O-8010) for another 24 hours. Cells were then fixed with 4% paraformaldehyde in PBS pH 7.2 overnight and immunostained as described above using mouse anti DM1A which binds to the tubulin alpha subunit (Neo Markers Cat #MS-581-P) as the primary antibody and donkey anti-mouse conjugated to Cy3 (Jackson Immunoresearch Cat #715-165-150) as the secondary antibody.

2.2.7 - Transmission Electron Microscopy (TEM)

Cells, plated and transfected in 6-well plates, were fixed forty eight hours post transfection using 2.5% glutaraldehyde in coccadylate buffer (pH 7.2, Electron Microscopy Sciences). The cells were washed in phosphate buffered saline (pH 7.2) and post fixed with 1% osmium tetroxide (Electron Microscopy Sciences). Dehydration was done through an increasing ethanol concentration and transitioned using propylene oxide. Spurr resin (Electron Microscopy Sciences) was mixed according to the manufacturers instructions. The cells were infiltrated into Spurr resin with a 1:1 Spurr propylene oxide mixture for 1 hour followed by pure Spurr overnight at 4°C. The following day, the cells were pelleted at the bottom of BEEM capsules, covered with pure Spurr resin and

polymerized overnight at 60°C. The cured blocks were trimmed and sectioned on a Sorvall MT-6000 ultramicrotome first using a glass knife followed by a diamond knife (Micro Star Technologies). Sections were collected on 200 count copper grids and contrasted with uranyl acetate and lead citrate. Images were captured using a Philips CM-100 TEM equipped with Motic 5.0 digital camera.

2.2.8 - Scanning Electron Microscopy (SEM)

The cells were plated and transfected on sterile 25mm coverslips placed in 6 well plates. The coverslips were fixed and dehydrated the same as TEM samples up to the propylene oxide step. Rather than infiltration of resin, the coverslips were placed in fresh propylene oxide and covered with aluminum foil. Small holes were poked into the foil to allow for slow evaporation of the propylene oxide, therefore totally dehydrating the tissue without causing the cell structure to collapse. The dried coverslips were mounted onto aluminum stubs with carbon adhesive tabs (Electron Microscopy Sciences). Samples were gold coated using a Hummer IV sputtering system for 5-10 minutes at 10-15 mA. All samples were imaged on a Topcon ABT-32 SEM equipped with the Printface image capture card and software.

2.2.9 - Apoptosis Induction

In order to compare the blebbing events seen in tau expressing cells, apoptosis was induced in non-transfected cell cultures. Cells were plated on sterile 2.5mm round cover slips (Belco Glass) in 6 well plates. The following day, the cells were exposed to 100nM taxol and allowed to survive for 48 hours. The taxol treated media was changed at

the 24 hour mark. At 48 hours, the cover slips were fixed with 2.5% glutaraldehyde in coccadylate buffer. The cells were processed for SEM and imaged as previously stated.

2.2.10 - Thioflavin staining

CHO cells were plated in eight well chamber slides (Lab-Tek II) and transfected the following day with PH-tau and wild type tau as stated above. Forty eight hours after transfection, the cells were fixed with 4% paraformaldehyde overnight and immunostained as described above using they were washed with PBS, permeabilized with 0.1% Triton X-100 (Fisher Scientific Cat mouse anti tau (DA9) (a gift from Dr. Peter Davies) as the primary antibody and donkey-anti-mouse-Cy3 (Jackson Immunoresearch Cat #715-545-150) as the secondary antibody.

After imunostaining, the slides were stained in 0.1% Thoflavin S (Pfaltz & Bauer Cat #T10955) in distilled water for 5 minutes, rinsed in distilled water, differentiated in 70% ethanol, rinsed again in distilled water and coverslipped with Vectorshield antifade mounting medium (Vector Laboratories Cat #H 1000). Cells were imaged using a Leica SP2 AOBS confocal laser scanning microscope. Images were analyzed using ImageJ.

2.2.11 - Determination of Caspase3 Activation

Cells were plated into 8- well chamber slides (Lab-Tek II) and each well was transfected with the appropriate GFP-fused tau vector. 48 hours post transfection, cells were fixed with 4% paraformaldehyde overnight and were imunostained as described above using rabbit anti active caspase 3 (Millipore Cat #AB3623) as the primary

antibody and Alexa Cy3 (Jackson Immunoresearch Cat #715-545-150) as the secondary antibody.

The slides were imaged on a Nikon Eclipse Ni-E equipped with Stereoinvestigator (MBF Bioscience). The system was used to collect the three different channels; GFP-fused tau, Cy3-caspase3, and DAPI nuclei. These images were opened in ImageJ and the cells labeled with the different colors were counted. Numerical data was tabulated and analyzed using Microsoft Excel.

2.3 - Results

In all experiments conducted, two vectors were tested in each condition. One vector contained pseudophosphorylation at T212, T231, and S262, while the second had the same sites along with the R406W mutation, one of those associated with frontal-temporal dementia. In all experiments, there was no detectable difference between those with and those without the R406W mutation. For continuity, all PH-tau images presented are images from the vector containing the R406W mutation.

2.3.1 - Okadaic acid treated wild type expressing cells causes similar pathological conditions as PH-tau

In order to be able to compare the ability of actually hyperphosphorylated tau to cause the same pathological condition in the cells as our pseudophosphorylation approach, okadaic acid was used to eliminate the function of the phosphatases in the cells, which should lead to wild type tau becoming hyperphosphorylated. Cells were

transfected with wild type fluorescent tau and exposed to okadaic acid for three hours prior to fixation at 24 hours post transfection.

The okadaic acid treated wild type tau expressing cells exhibited similar pathological structures as those transfected with the pseudophosphorylated constructs. Tau entered the nucleus and induced membrane zeiosis. Additionally, the cells were smaller in size and more rounded in shape, not well attached to the culture surface. In Figure 2.2A, the microtubules in an okadaic acid treated wild type tau expressing cell seems to be less arborous and are more focused into a ring-like structure around the nucleus. There also appear to be membrane blebs (Figure 2.2A, arrow) and a small, but easily noticeable presence of tau in the nucleus is detected.

Additionally, phospho-specific antibodies against phosphorylation at T212, T231 and S262 were used to detect phosphorylation at the specific amino acid resides on the protein. In Figure 2.2B-D, all three sites are depicted. Each of the three sites became phosphorylated when treated with okadaic acid.

2.3.2 - Live cell Imaging of PH-tau expressing cells shows a lack of microtubules and tau present in the nucleus

To further investigate the effect PH-tau had on cell structure and function, short-term live cell imaging was used in order to study the microtubule dynamics in PH-tau expressing cells as compared to wild type tau expressing cells. In these experiments, cells were imaged for several minutes with a frame taken at an average of every 3.5 seconds. When the wild type expressing CHO cell movies were analyzed, tau is associated with the microtubules and not at all present in any other part of the cell (Figure 2.3A). The

microtubules in these cells go through normal dynamic events of moving back and forth within the cell. The cells always maintain a healthy stretched-out, adhered appearance.

In the PH-tau expressing cells, microtubule dynamics were not detected. This was because microtubules were not visible in these cells at all (Figure 2.3B). In these cells, tau is found free-floating in the cytoplasm. Additionally, it was interesting to note that tau was also present in the nucleus of these cells. These cells are also more rounded in nature and seem to be less attached to the surface of the cover slip. The presence of intracellular organelles can be seen as a contrasting background within the green tau throughout the cytoplasm.

It is known that hyperphosphorylated tau sequesters normal tau and causes it to aggregate (Alonso et al., 2004). Additionally, it has been shown by Alonso *et al.*, that PH-tau binds to and sequesters healthy tau and causes the formation of filaments *in vitro* (Alonso et al., 2001c). In order to see what the effect PH-tau has on wild type tau in a cellular system, a co-transfection experiment was performed. To do this, a fluorescent tau vector was co-transfected with a non-fluorescent tau vector. Two conditions were used, one where wild type tau was fluorescent and another when PH-tau was fluorescent. The movies from this experiment (Figure 2.3C & D) showed that in the presence of PH-tau, wild type tau does change its cellular distribution and these cells do have a more sparse microtubule network when compared to cells expressing only wild type tau (Figure 2.3C & D). In cells expressing both tau constructs, the wild type tau is now also able to partially enter the nucleus and is seen free-floating in the cytoplasm, since in one condition, wild type tau is fluorescent and there is detectable fluorescence in the nucleus (Figure 2.3C). Although, these pathological situations were detectable, tau bound to

microtubules was also seen, unlike in the PH-tau only expressing cells (Figure 2.3C). In the case of the fluorescent PH-tau (Figure 2.3D), the cells are also blebbing at the membrane. Based on these images that there is an interaction of PH-tau and wild type tau and that this interaction triggers a cascade the causes the wild type tau to behave pathologically.

2.3.3- PH-tau cells are smaller in size than those expressing wild type tau

Through all of the live imaging, it was observed that PH-tau cells were smaller in size than wild type cells. It was suspected that this was due to the excessive membrane blebbing that was exhibited by PH-tau expressing cells. Images of the cells were taken and a perimeter of the cells was calculated using ImageJ and the data is presented in Figure 2.4. It was seen that the PH-tau cells are in fact smaller than the wild type cells. The bar graph represents the average perimeter of the CHO cell along with the standard deviation. The scatter bars plot the point where each of the counted cells lie. There is a range of sizes in wild type cells and non-transfected cells and it is interesting to note that the size of the cells transfected with wild type tau is larger than cells that are not transfected at all. It is believed that this is probably because of the additional microtubule stabilization in the tau expressing cells.

2.3.4 - Surface analysis of PH-tau induced membrane blebbing

In order to better visualize the surface of the cells to further investigate the membrane blebbing, scanning electron microscopy was used (Figure 2.5). In panels A, cells expressing PH-tau can be seen. These cells are noticeably unhealthy 48 hours after

expression of the protein. The cells are rounded in shape and are no longer attached to the culture surface. The cells themselves are full of zeiotic membrane protrusions pinching off the surface of the cells (Figure 2.5, panel A insert). These protrusions seem to be accumulating in the surrounding area. It is interesting to note that although only approximately 20-50% of the cells take up the plasmid during transfection, but 75% of the cells are positive for the presence of membrane blebs.

The PH-tau expressing cells should be compared to Figure 2.5B, which are of cells that are transfected with wild type tau as well as Figure 2.5C, which are CHO cells not transfected at all. These cells are longer, have a more spread out shape and seem to be well attached to the culture surface. Although there is the presence of several rounded cells, 90% of these cells seem to maintain the healthy normal CHO cells configuration.

Previous studies demonstrate that a majority of cells expressing PH-tau are also caspase 3 and TUNEL positive (Alonso et al., 2010). The zeiotic process could be the result of apoptosis. To be able to compare apoptotic cells with those expressing PH-tau, normal CHO cells were exposed to taxol to induce apoptosis (Figure 2.5D). These cells were much less healthy than the wild type and non-transfected cells, but they were not blebbing to the extent of the PH-tau cells. Dead cells can be easily detected in the culture, but there are still large populations of cells that are wide, elongated and well attached to the culture surface. This suggests that the PH-tau is the reason for the advanced and excessive zeiosis.

To gain quantitative measurements of blebbing cells in the wild type tau expressing cells as compared to PH-tau expressing cells, 300 combined cells from two separate SEM samples were scored as either positive or negative for membrane blebbing.

The results are shown in Figure 2.6. Blebbing is a normal cellular process and can be seen in 10% of the cells in the culture transfected with wild type tau. When the culture is transfected with PH-tau, the level of cells exhibiting membrane blebbing is over 70%.

2.3.5 - Ultrastructural Characterization of Zeiosis and Intracellular Degeneration

In order to study the intracellular degeneration and the blebbing that is occurring, transmission electron microscopy was done. In Figure 2.7, two healthy cells are shown, A is a cell from the culture that was transfected with wild type tau while B is of a normal, non-transfected CHO cell. Both of these cells are spherical due to the processing being done free-floating. The nucleus is defined without any inclusions and the cytoplasm is even with a large number of mitochondria. These are the cells to which the remaining electron micrographs should be compared

As the live cell imaging demonstrated, there are two different types of membrane blebbing seen in PH-tau transfected cells; a large bleb (Figure 2.8A arrow) and a small bleb (Figure 2.8B arrow). Additionally, in Figure 2.8A, you can also see a dark, electron dense intracellular/intranuclear inclusion. This structure has been seen in several cells with these large zeitoic protrusions. These inclusions are not seen in cells with small blebs or cells with wild type tau transfection. These blebbing cells usually also contain a larger number of intracellular vacuoles which contain a sizeable, electron dense material that usually takes up most of the vacuole.

Cells with intracellular degeneration can also be seen (Figure 2.9A & B). In the particular cell in Figure 8, there is a large amount of degenerated material in the center of the cell. This cell has many mitochondria that appear to be healthy, but also has the presence of vacuoles. The degenerating mass seems to be at the center of the cell in the

region where the nucleus is present. These zeiotic processes and intracellular degeneration are not present in the wild type transfected cells and the non-transfected CHO cells. Figure 2.9B shows a higher magnification of the intracellular degeneration. This is characterized by the accumulation of disorganized material in the cell. This degeneration is often seen in the nucleus, but can be present in other areas of the cells. There also seems to be the presence of aggregated filaments within the mass (as denoted by the box in the figure).

Figure 2.10A - C are higher magnification micrographs of protein aggregation in the vicinity of dead cells. The accumulation of aggregated fibers and fibers interacting with one another are shown with an arrow. This material was detected in a cell that seems to have recently died and broken open exposing the surrounding cells to this material. Figure 2.10C shows these fibers interacting with the surface of a zeiotic cell. These fibrils have the classic shape and appearance that matches aggregated tau as is forms paired helical filaments and straight filaments.

2.3.6 - PH-Tau Cells are Positive for Thioflavin S staining

Thioflavin S staining is used to detect protein aggregation in cells and tissues. This technique was used to determine if there was aggregated protein in the PH-tau transfected cells. The presence of positive thioflavin staining is seen in the PH-tau cells but not in those expressing wild type tau (Figure 2.11). Based on this data, this also suggests that tau is aggregated both inside the nucleus as well as in the cytoplasm.

2.3.7 - Caspase 3 seems to be active in non-trasnfected cells when PH-tau is transfected into the culture

It has been shown before that cells expressing PH-tau have a high probability of also expressing active caspase 3 and being positive for TUNEL staining. It has been noticed that not only do the cells expressing PH-tau have an increase in caspase 3 activation, but that the surrounding cells not expressing the pathological tau protein also seem to have active caspase 3 (Figure 2.12). Further work will be done to establish why this is occurring. More experiments need to be done to further explore this finding.

2.4 - DISCUSSION

Much of what is known about hyperphosphorylated tau's ability to be toxic stems from the de-stabilization of the microtubule network as well as its ability to form intra-cellular aggregates. The breakdown of the microtubule network leads to the inability of cells to traffic vital organelles and packaged neurotransmitters to the axon terminal. Without these necessary components, the neuronal signals are not transmitted and therefore, the former connections are not necessary, which leads to the retraction of neurites. This lack of signaling is the cause of the major loses axons and dendrites within a degenerating brain. Additionally, It is the accumulation of non-functional tau, both monomeric and aggregated that leads to eventual cell death. There are studies that support that it is aggregated tau in the form of paired helical filaments, which causes a cell to die (Cummings et al., 1998). There has been additional data suggesting that it is monomeric tau that causes the cell death seen in the various tauopathies (de Silva and Farrer, 2002).

The experiment that was carried out to test the ability of PH-tau to sequester normal tau proved to be a great model to visualize this process in cells. Figure 2.13 summarizes the findings presented here. It was seen that the fluorescent wild type tau, in double transfected cells also expressing dark PH-tau, the cells did not appear as the healthy, wild type expressing only cells. There was the presence of a microtubule network, which was not detected in the PH-tau expressing cells alone. However, there was also the presence of tau free floating in the cytoplasm and, to some degree, the presence of tau in the nucleus as well. This suggests that there was some interaction between healthy tau and PH-tau because now the wild type tau was behaving to some extent the same as the PH-tau.

Even with the aggregation of hyperphosphorylated tau and its sequestering of healthy tau, these are most likely not the only mechanisms leading to cell dysfunction and eventual death. There are other events that lead to cell dysfunction and it is these events where tau interacts with different cellular components and cause damage through other pathways in cells. This work discusses the process of zeiosis, or excessive membrane swelling and pinching off. There has been proof of membrane destabilization leading to cell death in spinal cord injury (Zhang et al., 2006). The loss of membrane stability causes all of the internal membrane bound organelles such as the endoplasmic reticulum, mitochondria and the Golgi apparatus, to also no longer be stabilized. Without these functional organelles, the cells no longer posses the necessary inner components to carry out there required functions. Other studies have shown such situations, for example, it has been sown in other studies that mitochondrial destabilization leads to neurodegeneration (Karbowski and Neutzner, 2012). Future work will look into the positioning and the real

time movement of these organelles, as it is believed that there is a problem in the stabilization and the transport of these organelles throughout the cell somas. Additionally, use of cell lines such as PC12 cells, which can be differentiated into neurons, would prove useful to track transport of this material through an axonal-like projection.

More recently, it has been shown that tau not only interacts with the plasma membrane, but that the hyperphosphorylated form also contributes to the disruption of the cell membrane (Jones et al., 2012; Pooler and Hanger, 2010). Jones has shown that tau interacts with anionic lipids and triggers protein aggregation. Additionally, membrane permeabilization is a proposed participant in the mechanism that makes aggregated tau toxic.

Additionally, the blebbing of the cells seems to cause a decrease in the overall size of the cells. This decrease in size will also provide less room for the cellular components to be housed as well as additional, and smaller organelles may be lost during the blebbing process. It has been shown through detailed stereological investigations of the entorhinal cortex of Alzheimer's disease patients post-mortum that the cells of this region tend of be smaller in size and irregularly shaped (Artacho-Pérula, 2007). The CHO cells investigated in this work also appear to have the same decrease in size and the irregular shape. The CHO cells expressing the PH-tau were rounded, and often not well spread out and not adhered well to the culture surface.

There was question as to whether or not the cells were undergoing apoptosis and if the blebbing was just a product of the apoptotic mechanism that has already been proven to be occurring. To test this, cultured CHO cells without any transfection were exposed to taxol for 48 hours. Taxol, a toxin that is responsible for the permanent

polymerization of the microtubule network, would lead to apoptosis activation. These cells were imaged under the scanning electron microscope. The image did show rounded cells that seemed to be dead. These cells, however, did not express the blebbing to the same extent that the PH-tau expressing cells did. This support the idea that the zeiotic process that the cells are undergoing is mediated by the tau directly and not by an apoptotic mechanism.

The transmission electron microscopy allowed for the visualization of the internal components of the zeiotitic blebs. These samples demonstrated that the inside of the blebs are electron lucent and clear of any aggregated material or debris. Additionally, the electron microscopy also allowed for the interior of the cells to be seen and analyzed demonstrating that some of the material seen in the movies were not just healthy organelles, but also degenerated structures within the cells. The cells contained many vacuoles, suggesting that the cells were in the process of trying to break down internal that could be causing internal harm. The mitochondria appeared to be visually healthy, but the detection of larger membrane bound organelles were not detectable. With the approach that was accomplished in this study, the cells were not able to be positively distinguished for the presence of tau, so future work will focus on the use of immune-gold labeling of these cells to see both which cells are expressing tau and where in the cells that tau is residing.

The cells expressing the PH-tau constructs in this study also came up positive for intracellular aggregates by thioflavin S staining. This positive result is further supported through the detection of fibrils under the transmission electron microscope. This data is very interesting because it begins to shed light on which specific sites of phosphorylation

are important to drive tau aggregation. Furthermore, the presence of aggregated protein is co-localized with tau expressing cells when immunocytochemically stained for the presence of tau and post-immuno-stained with thioflavin S. Initially, it was not seen that the non-fluorescent tau vectors also caused tau to enter the nucleus.

Finally, it was noticed that in cells surrounding PH-tau expressing cells, that there were a large population of tau negative cells also expressing caspase 3 activation, when stained with an active caspase 3 antibody. Future work will explore this phenomenon further. The blebbing of the membrane may be a survival mechanism of the cells to remove pathological tau from the soma. In the process, the cells are then releasing membrane blebs that are full of pathological tau. As mentioned before, there have been studies that have shown that pathological tau could sequester normal healthy tau. If other cells were to take up these blebbed-off vesicles, they could expose their healthy MAPs to pathological tau and cause a sequestration of the protein. This potentially supports the hypothesis that tau is a prion-like protein and has the ability to spread from cell to cell and sequester normal, healthy MAPs, leading to a pathological condition in otherwise healthy cells. There is ever increasing evidence that tau can function like a prion (Hall and Patuto, 2012). Kfoury and colleagues have shown that tau pathology can be spread when surrounding cells in culture pick up aggregated tau in cell culture (Kfoury et al., 2012). Furthermore, it has been demonstrated that tau associated with exosomes may play a role in disease transmission (Saman et al., 2012). This type of transmission also seems to be apparent in amyloid beta mediated degeneration (Jucker and Walker, 2011).

Taken together, this work may begin the shed light on the specific resides of phosphorylation that mediate tau's ability to become prion-like in nature. Further work will be conducted to develop a study to see if, in fact, this is the case.

2.5 - CONCLUSION

This work has demonstrated that when the amino acid residues T212, T231 and S262 of tau protein are hyperphosphorylated, with or without the R406W mutation, this leads to several cellular pathologies other than microtubule disruption. These pathologies include membrane zeiosis, intracellular vacuole formation, protein aggregation, and tau translocation into the nucleus.

The use of a pseudophosphorylated tau could raise question if the pathologies seen can in fact be caused by actual phosphorylation at these sites. The use of okadaic acid on wild type tau demonstrated that actual hyperphosphorylated tau leads to the same pathological cellular responses, suggesting that pseudophosphorylation, at least at the sites studied here, is a successful model to begin to study tau hyperphosphorylation. Furthermore, the use of phosphor-specific antibodies demonstrated that each of the three above mentioned sites were phosphorylated when treated with the okadaic acid.

Membrane blebbing is a normal and healthy occurrence that occurs in many cell types. Usually, these blebs are taken back into the cell. It is evident that when cells are expressing PH-tau, these blebs are released fro the cells into the extracellular environment. This was detected through scanning electron microscopic imaging. Furthermore, it was noticed that these zeiotic blebs are found throughout the culture environment. Additionally, the presence of tau negative, but caspase positive cells,

suggests that there is some form of disease progression as cell death is being transferred to other, non-transfected cells in the culture. This is in support of the prion-like nature of tau protein, which has been shown in several other studies. Further work will be done to investigate the ability of this specific form of pseudophosphorylated tau to be able to transfer the toxic condition through spread of the pathological protein.

The presence of positive thioflavin staining is confirmatory for the presence of aggregated protein, which was confirmed using the transmission electron microcopy. The phosphorylation at the three mentioned sites seem to drive the aggregation of tau protein. Further work will be done to investigate if this aggregation is cytotoxic itself, or if it is the monomeric tau that is, in this case, the toxic molecule.

It was also noticed that PH-tau enters the nucleus. Further work will be needed to investigate the mechanism by which tau enters this, otherwise tau-free space within the cell.

Taken together, these results suggest that the de-stabilization of the microtubule network not only leads to compromised cellular transport, but that there are other pathologies able to be detected, both related to and those not related at all to microtubule disruption. Further work will be needed to investigate the mechanisms by which PH-tau plays a role in all of these different cellular conditions.

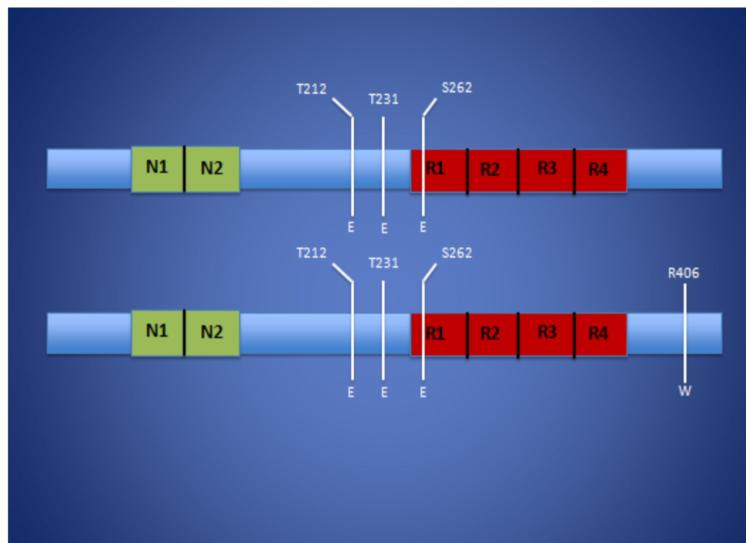


Figure 2.1 - Generation of PH-tau constructs using site directed mutagenesis: 2N4R wild type human tau had amino acid residues T212, T231 and S262 switched to a glutamic acid to mimic the change in charge distribution that phosphorylation at these sites would cause. Additionally, one of the mutations (R406W) associated with Frontal-Temporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) was used to see if the inherited forms play a major role in the development of tauopathies. In this work, the presence of the R406W mutation did not change the progression of the analyzed pathologies.

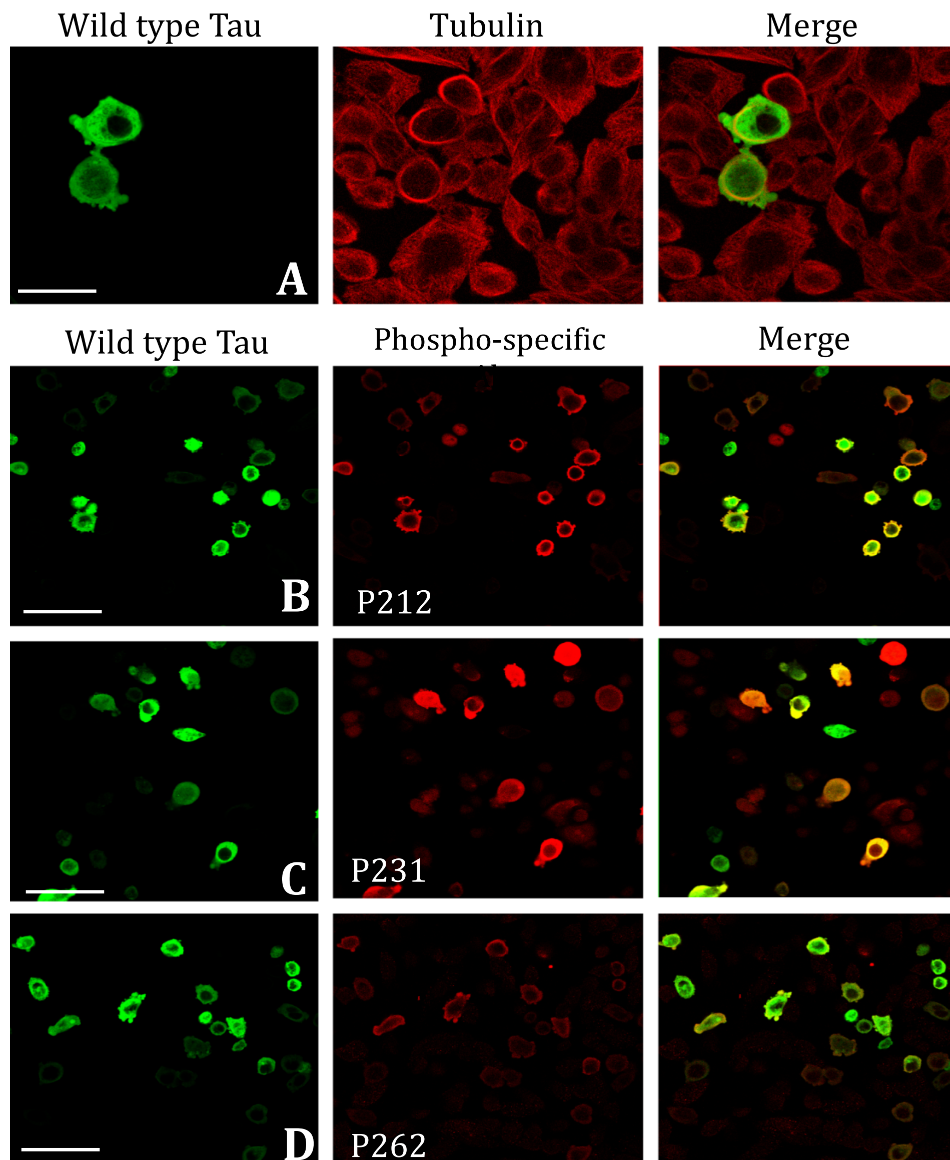
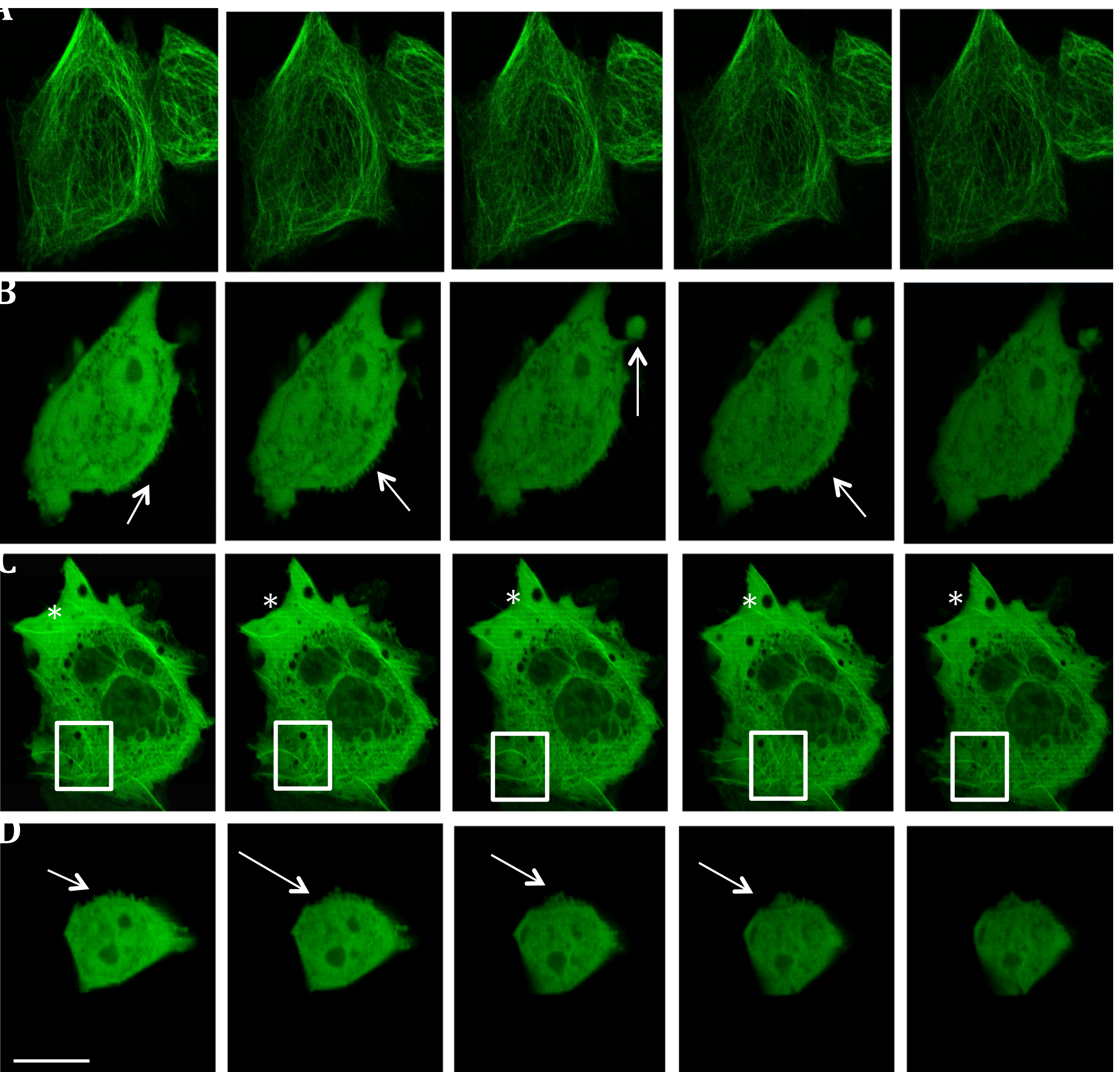


Figure 2.2 – Okadaic acid treatment of wild type tau expressing cells yielded much of the same pathology as the pseudophosphorylated PH-tau expressing cells: CHO cells were transfected with wild type tau only. 3 hours prior to fixation, the cells were exposed to okadaic acid and immunostainined accordingly. This experiment demonstrated that the tubulin cytoskeleton formed a ring around the nucleus, a common feature seen in PH-tau expressing cells (A). Additionally, the cells appeared to be blebbing at the membrane and tau seemed to enter the nucleus (A). Here it is shown, using phospho-specific antibodies, that all three of the pseudophosphorylation sites that will be used in this study are phosphorylated when treated with okadaic acid and this phosphorylation leads to the pathology seen in PH-tau expressing cells. These results suggests that these sites play a major role in driving the degenerative pathology seen in the pseudophosphorylated tau expressing cells (B-D). Scale bars A: 20um, B-D 50µm



Wild type
Tau

PH -Tau

D PH –Tau
F wild type
Tau

F PH –Tau
D wild type
Tau

Figure 2.3 – PH-tau causes membrane zeiosis and tau free floating in the cytoplasm: These are images taken from CHO cell transfected with wild type tau (A) , PH-tau (B), co-transfections of fluorescent wild type and dark PH-tau (C) and dark wild type and fluorescent PH-tau (D). (A)Wild type tau binds well to the microtubule network and is not found any other place inside the cell body. (B) In cells transfected with PH-tau, tau no longer associates well with microtubules, but is rather diffused throughout the cytoplasm and enters the nucleus. The membrane in PH-tau expressing cells is blebbing off (arrows). (C) The co-transfection demonstrates that PH-tau does sequester normal tau. In cells expressing both vectors, there was a healthy microtubule network present (frame), but it also has an increased amount of tau in the cytoplasm when compared to wild type alone, the presence of large intracellular vacuoles (asterisk) and a small amount of tau in the nucleus. In C, the fluorescent tau in this experiment is wild type, this protein must in some way be influenced by the toxic PH-tau. In row D, the fluorescent tau is PH-tau and this cell exhibits membrane blebbing and tau in the nucleus as does the PH-tau alone. Scale bar 10μm

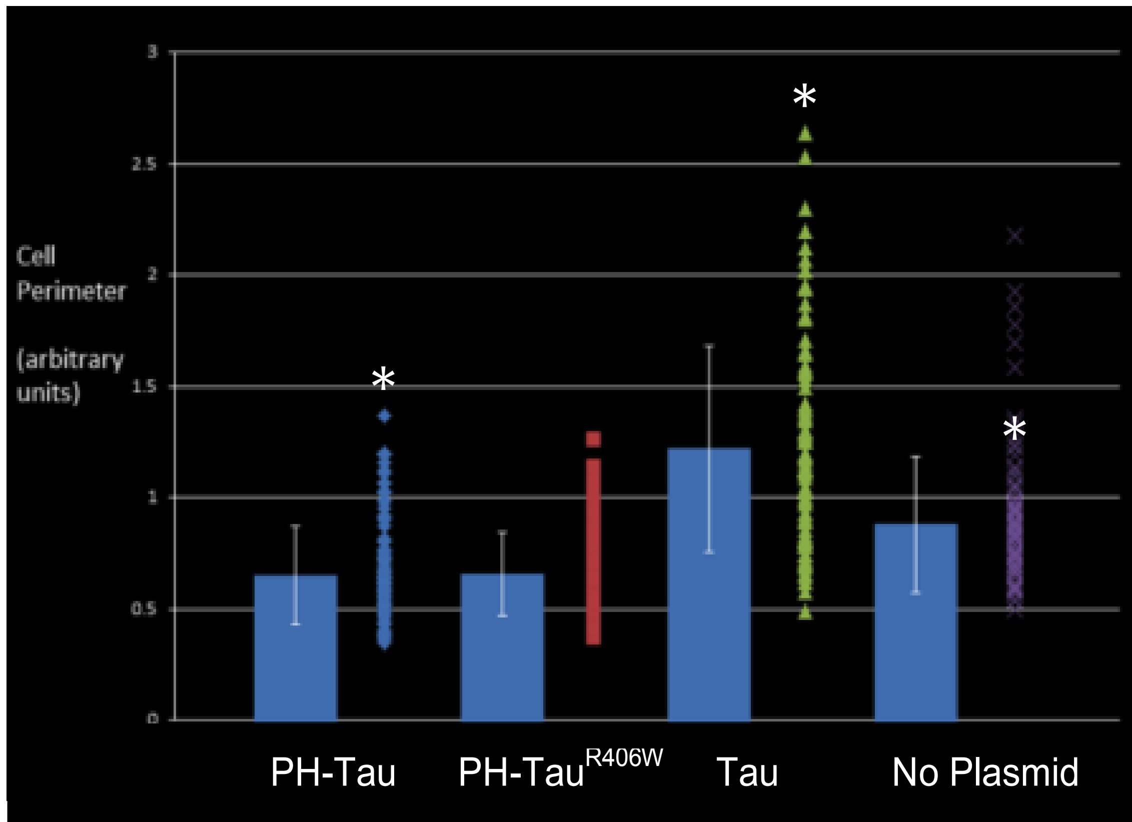


Figure 2.4: PH-tau expressing cells are smaller in size than wild type tau expressing cells: Cells were transfected with with fluorescent wild type or fluorescent PH-tau and images were captured at low magnification. The perimeter of one hundred cells were measured using arbitrary units in imageJ. The scatter graph shows the distribution of all one hundred points. The bar graph shows the average perimeter and the standard deviation of the cell perimeters. These measurements show that the PH-tau cells are significantly smaller than those transfected with wild type tau. It is also interesting to note that CHO cells without transfection (no plasmid) are significantly smaller than cells transfected with wild type tau suggesting that tau may play a role in allowing CHO cells to increase their size because of extra microtubule stability.

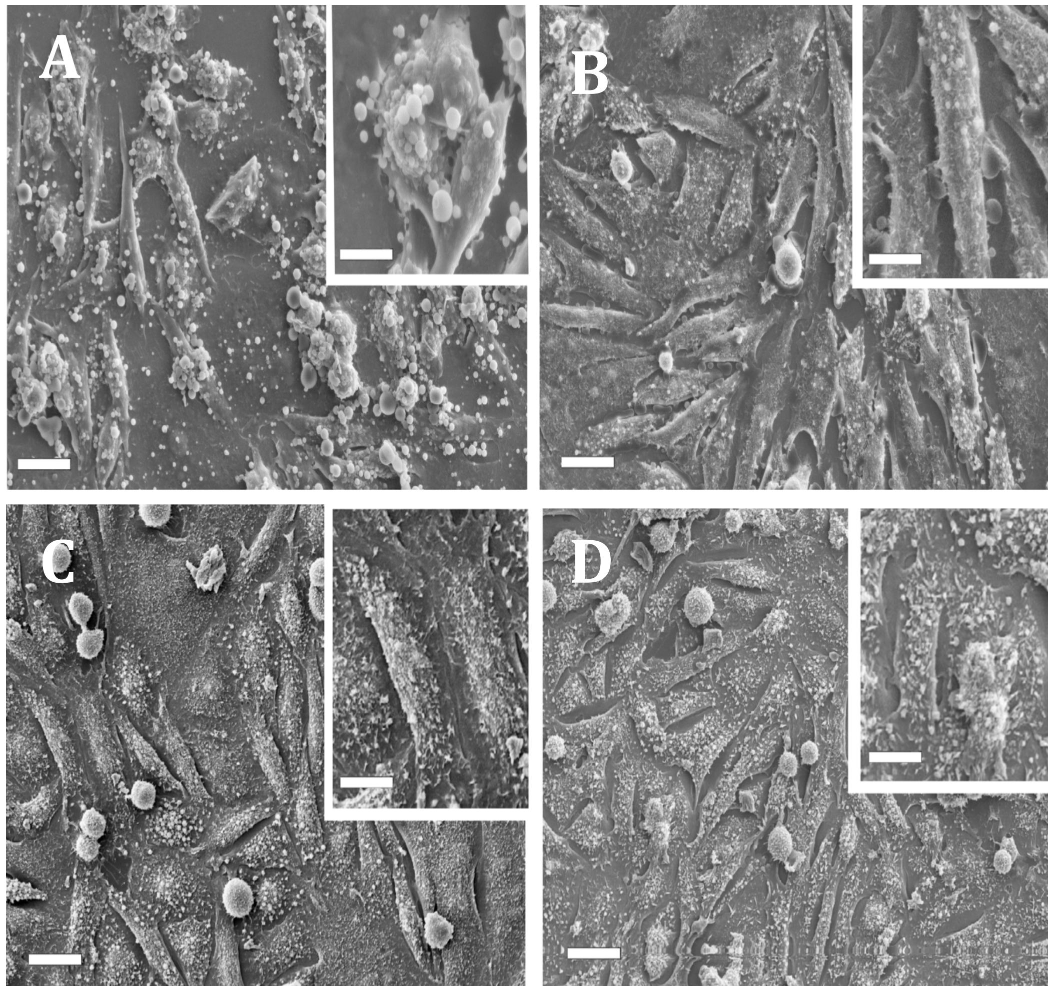


Figure 2.5 - Zeiotic blebs are detected using SEM in PH-tau expressing cells and accumulate in the culture environment: All cells were fixed 48 hours after transfection with PH-tau (A), most of the cells are rounded up and no longer attached to the surface of the culture vessel. There are many membrane protrusions on the cell surface as well as in the surrounding area. When cells are transfected with either wild type tau (B) or when the cells are not transfected at all (C) they appear to be nicely adhered to the culture vessel and there is no evidence of membrane zeiosis. The cells also appear to present a healthy CHO cell morphology, where they are longer and spread out. CHO cells were also exposed to taxol for 48 hours prior to fixation to image the cellular morphology in an apoptotic state (D). The cells, while more rounded up, do not display the same zeiotic events seen in those cells expressing PH-Tau. All scale large image scale bars are 15 μm, insert scale bars are 4 μm).

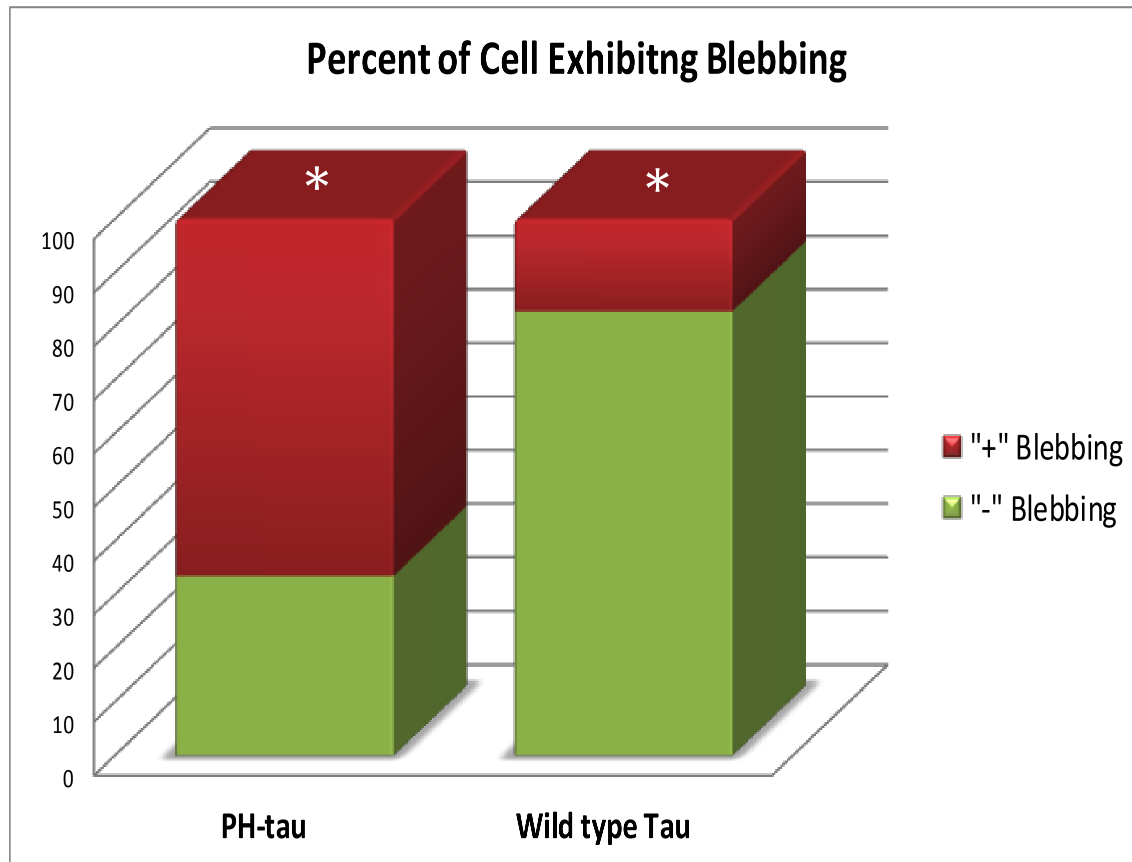


Figure 2.6 - Quantification of cells exhibiting membrane blebbing increases drastically in PH-tau positive cultures: Transfected cells that were used for scanning electron microscopic analysis were scored. 300 cells between two different SEM samples were counted and scored for either being positive for membrane blebbing or negative for membrane blebbing. The level of membrane blebbing significantly increased (60%) in those cultures transfected with PH-tau when compared to those transfected with wild type tau.

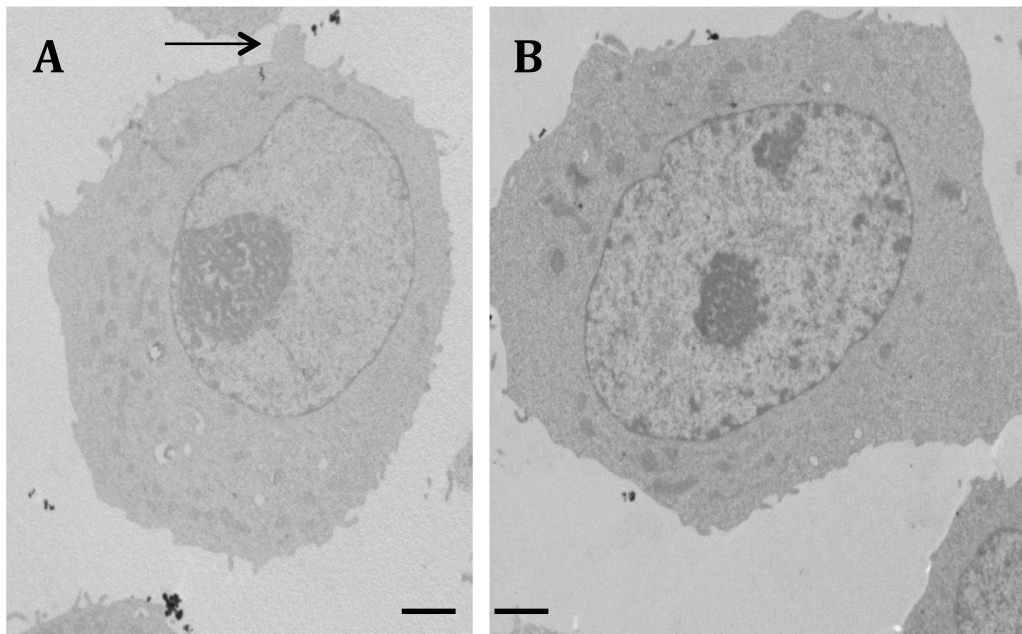


Figure 2.7 – Ultrastructural analysis of CHO cells transfected with wild type tau and without transfection: Cells were transfected with either wild type tau (A) or left without any plasmid introduction (B) and processed for standard biological transmission electron microscopy. These cells are very healthy CHO cells. They were processed free floating, which will account for their rounded appearance, rather than the classical adherent shape seen in the SEM images. The cells have a well defined nucleus and exhibit only minor, or normal, membrane blebbing as seen in panel A (arrow). The cytoplasm contains healthy mitochondria and do not show an excessive amount of vacuoles present. Scale bars: A - 1µm, B – 500nm

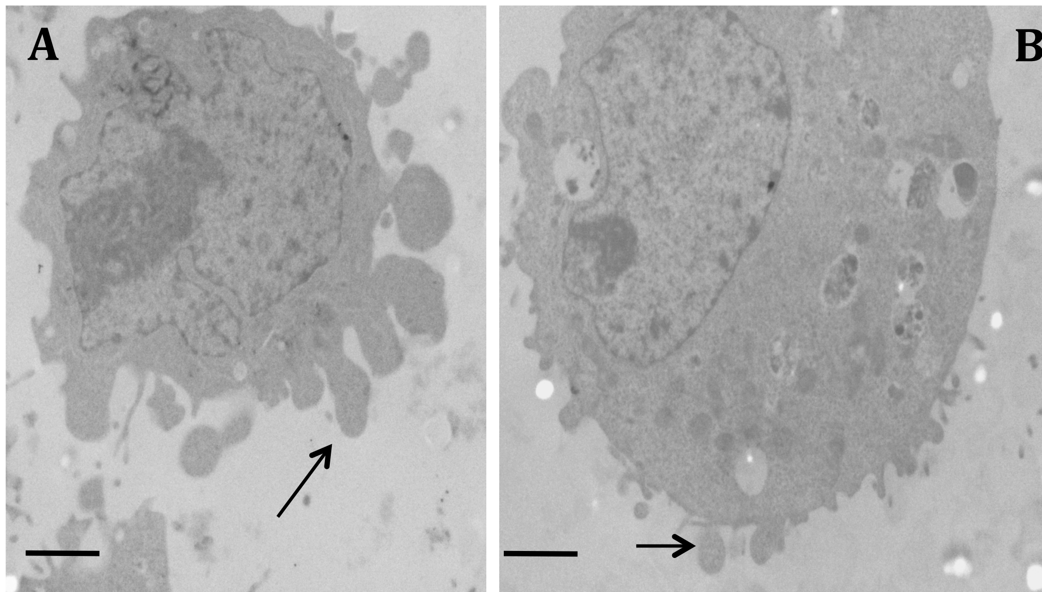


Figure 2.8 – Ultrastructural analysis reveals membrane blebbing in PH-tau expressing cells: Cells were transfected with either PH-tau and processed for standard biological transmission electron microscopy. As was seen in the live cell imaging, the PH-tau cells seem to be loosing a large volume of cytoplasmic mass and membrane surface area through the process of zeiosis. Two types of blebbing has been detected, cells with large blebs (A) and cells with smaller blebs (B). The two types of membrane blebs are shown by arrows. These cells also appear to have the presence of excessive cytoplasmic vacuoles (B), a classical sign of internal toxicity. Additionally, the presence of extracellular debris is evident throughout the cultures of cells expressing PH-tau. Scale bars: 1μm

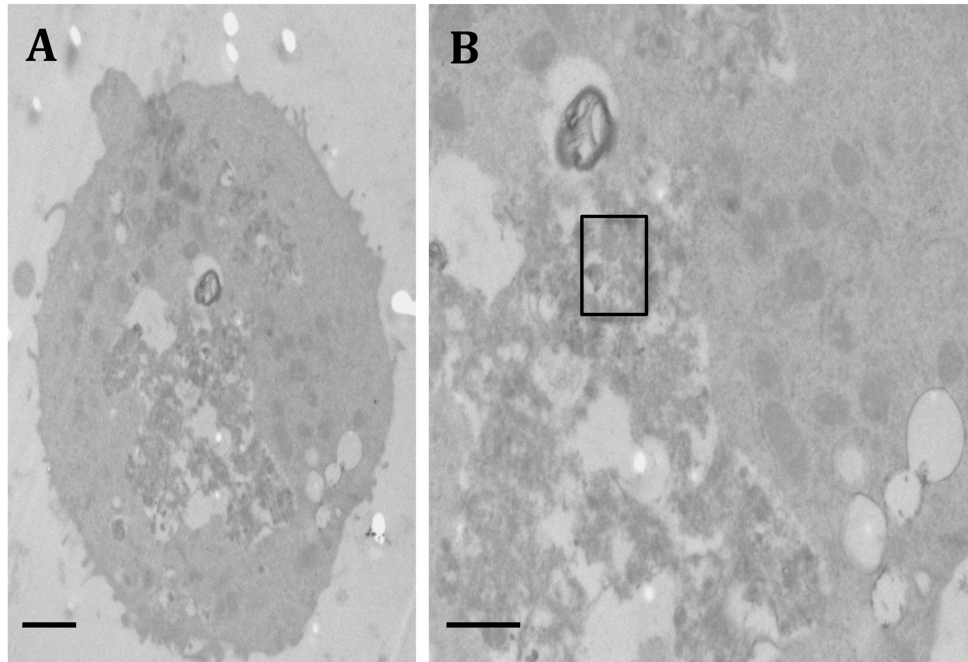


Figure 2.9 – Ultrastructural analysis intracellular degeneration: Cells were transfected with PH-tau and processed for standard biological transmission electron microscopy. In some cells, degenerating regions of the cytoplasm can be seen (A & B). The higher magnification (B) shows the internal structure of the cells (inside square). It is evident that the internal components of many of these cells is degrading. This could be due to a lack of cytoskeletal stabilization. Additionally, cytoplasmic vacuoles can be seen. Scale bars: A –1μm, B – 500nm.

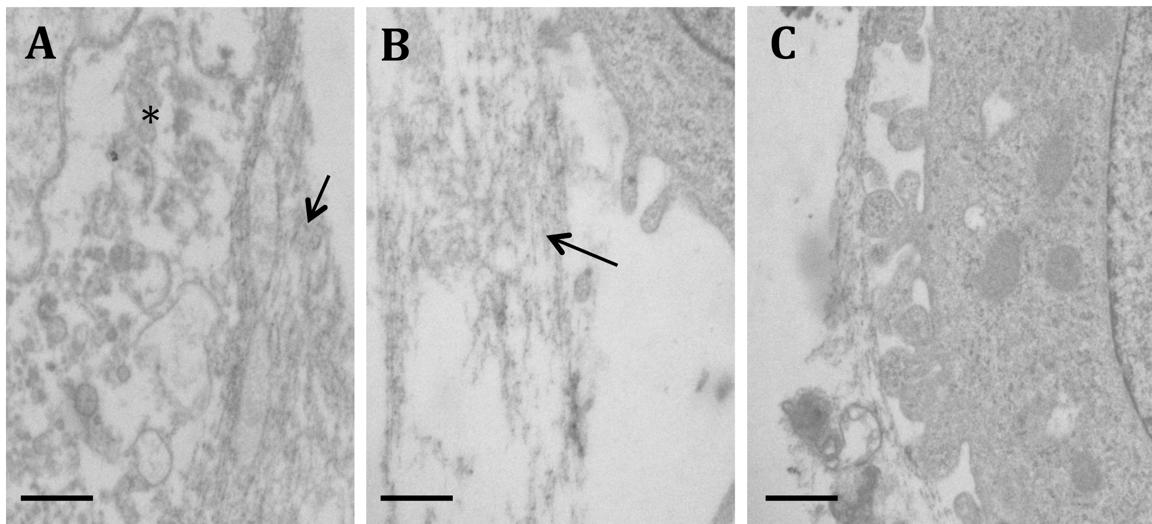


Figure 2.10 – Ultrastructural analysis reveals aggregated protein : Cells were transfected with PH-tau and processed for standard biological transmission electron microscopy. TEM also allowed for the visualization of aggregated, fibrous proteins (A-C, arrows). These fibers are often seen in dead, busted cells and also often associate with other cells, these cells are also often zeiotic (C). A higher magnification o the internal degeneration, shows that the cytoplasm in this region is not homogeneous and healthy, but is rather clumped and containing aggregated components. Scale bars: A – C: 500nm

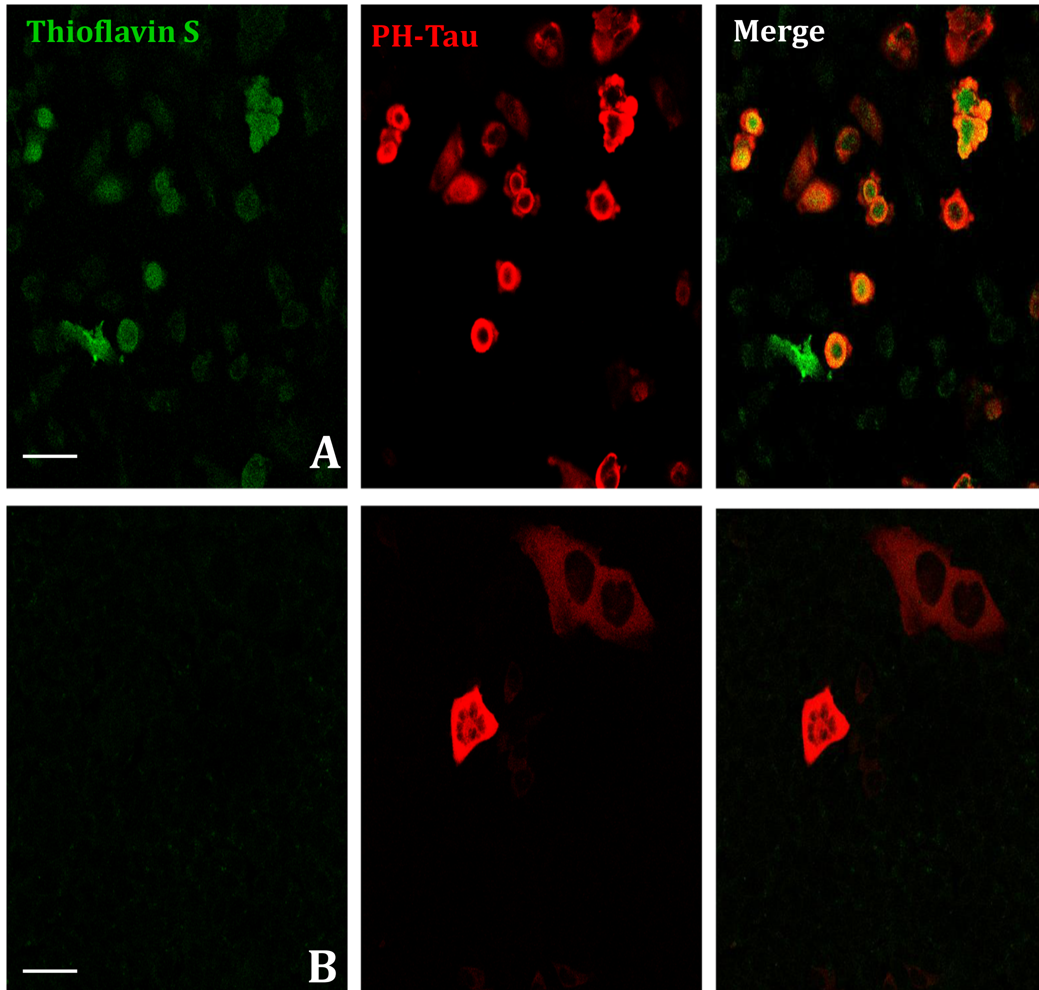


Figure 2.11 – PH-tau expressing cells are positive for thioflavin S staining: These cells were transfected with wild type or PH-tau, fixed 48 hours later and immunostained for tau followed by a counter-stain with thioflavin S. The cells expressing PH-tau were all positive for thioflavin S staining which supports the presence of aggregated protein within the cells (A). It is important to note that there is no background staining in non-transfected cells. CHO cells expressing wild type tau were did not stain positive for thioflavin S (B). This suggests that the presence of PH-tau causes protein aggregation in CHO cells. Scale bars: A-50µm, B-25 µm

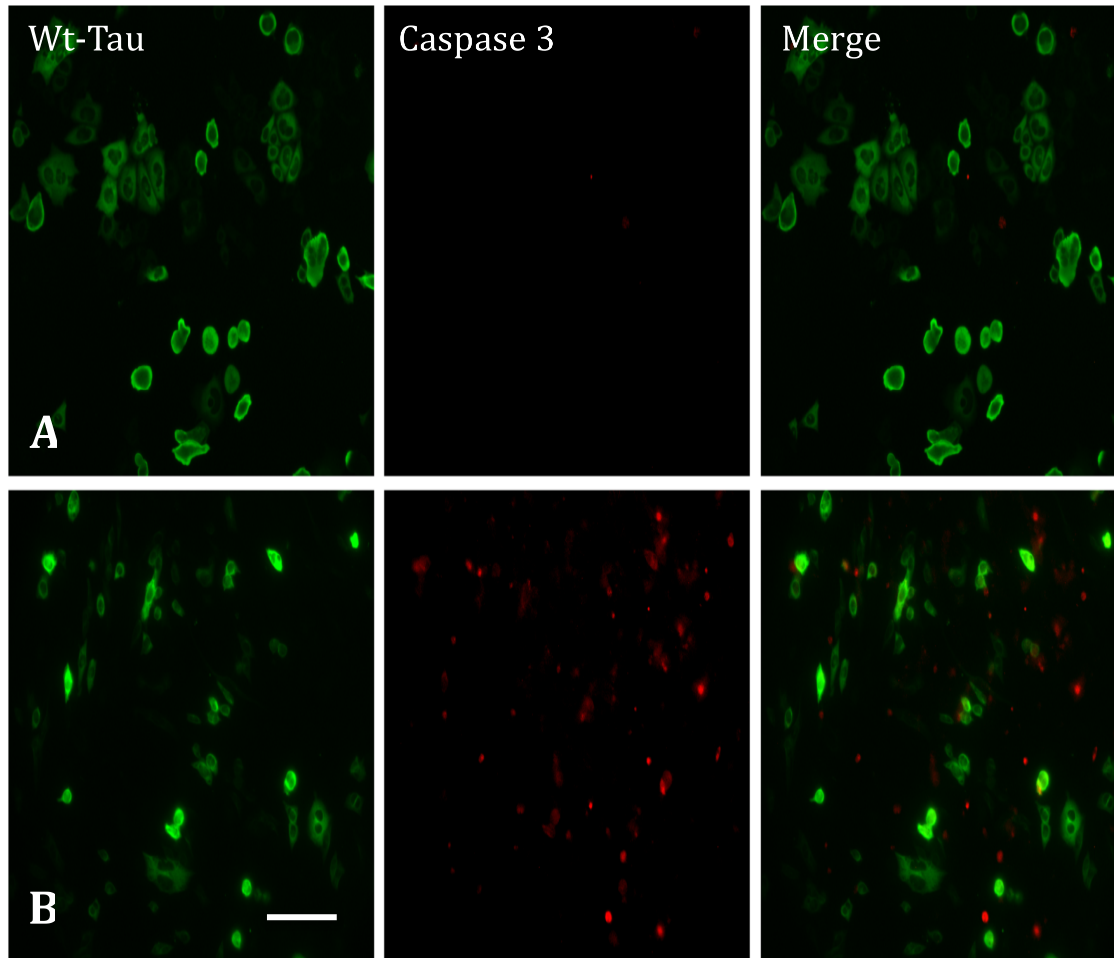


Figure 2.12 – Caspase 3 activation is increased in both PH-tau expressing cells and non-trasnfected cells in the culture: It has been previously reported that tau plays a role in caspase 3 activation. Here both wild type and PH-tau were transfected into CHO cells. 48 hours later, the cells were fixed and immunostained with anti-active caspase 3. It was seen that in the PH-tau cells, a large majority of the transfected cells were positive for active caspase 3 (B) when compared to the wild type expressing cells (A). What was particularly interesting was that there was a large majority of cells not transfected that came up positive for caspase activation in the PH-tau sample. Scale bar: 50µm.

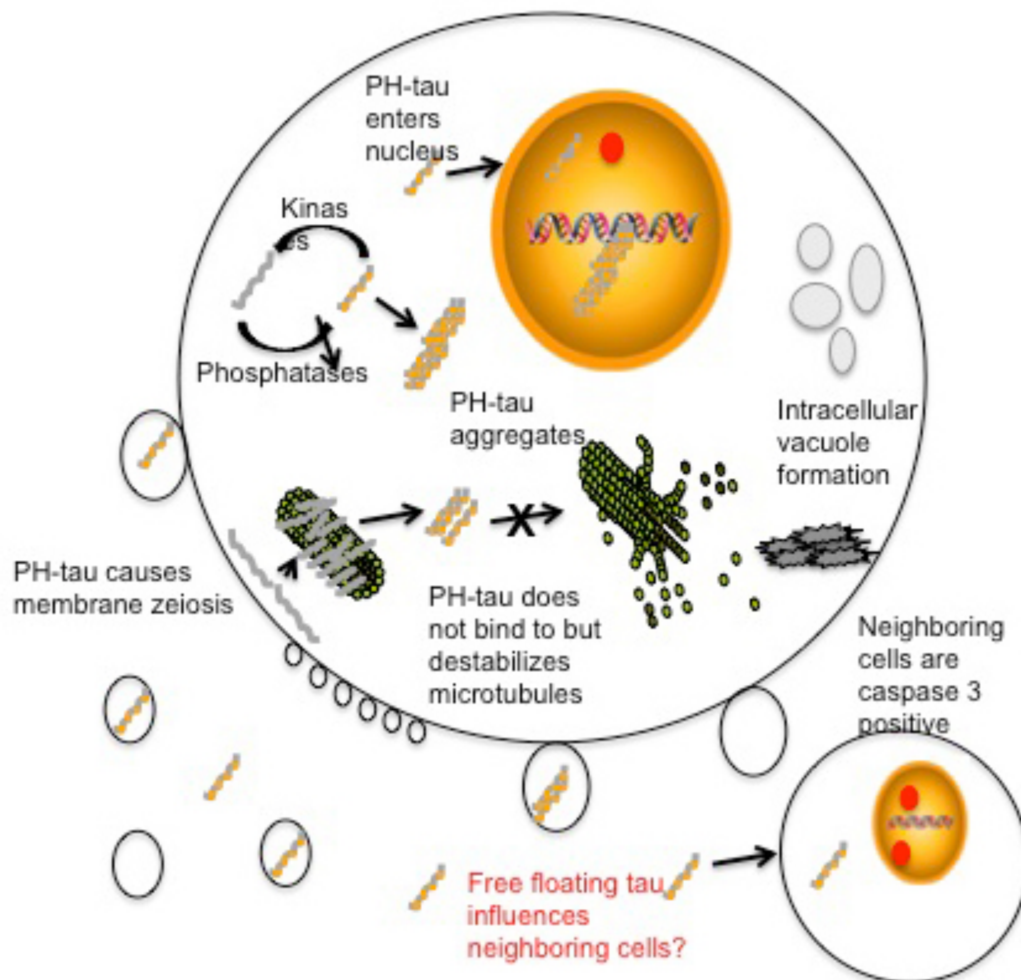


Figure 2.13 – Summary of Detected Pathology – This scheme depicts the cellular pathology caused by the expression of PH-tau. These include microtubule breakdown, tau aggregtion, caspase 3 activation in PH-tau expressing and neighboring cells and intracellular vacuole formation as well as membrane blebbing.

CHAPTER THREE

Effect of tau hyperphosphorylation on the actin cytoskeleton

Abstract: Tau is a microtubule associated protein (MAP) that is found in the neurons of the central nervous system. Hyperphosphorylation of tau causes it to no longer bind to and stabilize microtubules, but rather tau aggregates and forms neurofibrillary tangles. Actin microfilaments play a major role in the formation and stabilization of synaptic boutons. In Alzheimer's disease, these synapses are lost as the neurites are retracted due to the destabilization of the cytoskeletal network which leads to impaired signaling. Tau has been shown to interact with actin. To analyze the effect tau hyperphosphorylation has on the actin cytoskeleton, tau pseudophosphorylated at T212, T231, and S262 (PH-tau) was transfected into CHO cells. PH-tau appears to cause a major breakdown in the F-actin structure within the cells. These cells are either totally void of actin or have actin aggregated into puncta within the cytoplasm. This actin breakdown is also occurs in wild type tau expressing cells treated with okadaic acid to induce hyperphosphorylation, which supports the hypothesis that pseudophosphorylated tau is causing the pathologies seen in actual hyperphosphorylated tau. Currently, protocols to quantify the concentration of total F-actin and length of individual actin filaments do not exsist. Working with colleagues from the CUNY/CSI Computer Science Department, we are developing ImageJ plugins to facilitate these measurments. This work demonstrates that when PH-tau is expressed in cells, the level of tau is inversely proportional to the level of F-actin in the cells, where wild type tau . Interestingly, the level of total actin does not change between wild type tau and PH-tau expressing cells, suggesting that this lack of F-actin does not change expression, but rather only interferes with its polymerization. Further work to biochemically analyze the level of total and F-actin being expressed in the PH-tau and wild type tau transfected cells will be explored at a later time.

Keywords: tau, neurodegeneration, actin, Alzheimer's disease

3.1 - INTRODUCTION

Tau is the microtubule-associated protein (MAP) that is specific to neuronal cells. Tau is the primary protein that provides stability to the microtubules within the axons. The axonal microtubules are one of the cytoskeletal components responsible for both structural support as well as intracellular/intra-axonal transportation. Most of the necessary cellular components required at the axon terminal are created and packaged in the cell soma and require relocation to the axon terminal. These cellular components consist of mitochondria, which provide the necessary ATP for axonal signaling as well as packaged neurotransmitters to propagate signal transmission. It is the stabilized microtubule network that is used as the “highway” by which all of these molecules are moved from the soma to the axonal terminal via the movement of the motor proteins, dyanin and kinesin (Cho and Vale, 2012; Hirokawa, 2011). Without this cellular movement within neuronal cells, the axonal structure is compromised leading to a destabilized microtubule structure. This renders the cell unable to propagate the action potential and eventually a retraction of neurites and cell death.

Tauopathies are classified as a group of neurogenerative diseases that are caused by the dysfunction of MAP tau. This family of diseases includes disorders such as Pick’s and Alzheimer’s diseases. In above mentioned neurodegenerative disorders, tau no longer binds to microtubules, but rather forms both paired helical and straight filaments (Alonso et al., 2001a; Alonso et al., 2008; Goedert et al., 1992; Gomez-Ramos et al., 2004; Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Iqbal et al., 1986)). Together these filaments join together to form the neurofibrillary tangles, the classically seen intracellular protein aggregate characteristic to Alzheimer’s disease. In its pathological

state, tau no longer performs the function of microtubule stabilization, but rather aggregates with itself, leading to the formation of neurfribrillary tangles.

There are six tau isoforms seen in humans and are generated by alternative splicing of the pre-mRNA. These isoforms can include three or four microtubule binding domains, all which reside at the C-terminal domain as well as zero, one or two 29 amino acid N-terminal inserts (Goedert et al., 1989; Himmler et al., 1989).

There are several post translational modifications that occur on tau, which include acetylation (Irwin et al., 2012), glycosylation (Gong et al., 2005; Liu et al., 2004b; Smet-Nocca et al., 2011) and phosphorylation (Grundke-Iqbal et al., 1986b; Iqbal et al., 2009). All of these known post-translational modifications seem to contribute to tau pathology, but the best studied and most notable is phosphorylation. The change in the Tau protein that causes it to no longer associate with microtubules is hyperphosphorylation of the protein. At normal phosphorylation states, there are approximately 3 moles of phosphate per molecule of protein. In the hyperphosphorylated state, the protein carries around 8 moles of phosphate per molecule of protein (Köpke et al., 1993). When, hyperphosphorylated, certain additional sites that are not normally phosphorylated become so. These additional sites that are seem most phsohporylated in hyperphosphorylated tau are Thr212, Ser214, Thr231, Ser235, and Ser262 (Brandt et al., 2009; Drewes et al., 1995; Jenkins and Johnson, 1997; Sengupta et al., 1997; Singh et al., 1996). These sites are also shown to be involved in tau self assembly (Alonso et al., 2004).

Previous work has shown that *in vitro* that hyperphosphorylated tau induced self-assembly and the formation of filaments as well as the inability to promote microtubule

assembly (Alonso et al., 2008). It has also been shown that phosphorylation at Ser262 accelerates tau assembly into filaments.

The discoveries of mutations on the tau gene have demonstrated that mutations alone are enough to promote cell death and subsequently dementia in patients (Poorkaj et al., 1998; Spillantini et al., 1998). There are three reported types of tau mutations; missense (Chang, 2008), deletion (Kovacech and Novak, 2010) and intronic (Wang et al., 2010b). Missense mutations cause an amino acid residue to be switched while the mutations in the intronic region cause an overexpression of 4 microtubule binding domain tau. Patients with mutations causing Frontal-Temporal Dementia with Parkinsonism (FTDP-17) have displayed neurons containing neurofibrillary tangles and cell death. Previous work has shown *in vitro* that certain mutations associated with FTDP-17 has given tau the ability to self-assemble when containing approximately half the level of phosphate molecules as compared to wild type tau (Alonso et al., 2004). When tau is phosphorylated, there is most likely a conformational change that takes place that makes the molecule inhibitory and that this toxic state is more easily reached if the protein carries the FTDP-17 mutation (Alonso et al., 2004).

Our prior work has looked into the effect that specific sites of phosphorylation has on the Tau protein (Alonso et al., 2010). In order to mimic site-specific phosphorylation, a pseudophosphorylation model was utilized. To do this, specific serine and threonine residues on tau were switched with glutamic acid by site-directed mutagenesis. This amino acid switch mimicked the negative charge that would be added by the addition on a phosphate group at that site. We refer to this tau as pathological human tau (full length human tau with pseudophosphorylation sites T212, T231 and S262

(PH-tau). It was found that no single site, either on the wild type tau backbone or on the tau with the R406W mutation, was able to alter tau's normal function. In cells expressing this single pseudophosphorylation resides, the tau still bound tightly to the microtubules and the cells were healthy and there was no evidence detected to apoptosis markers active (Alonso et al., 2010).

Among several combinations of pseudophosphorylation sites tried without a toxic effect, the combination of T212, T231 and S262 caused tau to no longer associate with microtubules (Alonso et al., 2010). The site S262 alone and in conjunction with other sites caused tau to associate with microtubules more weakly. Specifically three pseudophosphorylation sites (T212, T231, and S262), both with and without the presence of the R406W mutation, caused tau to no longer bind to microtubules, but rather distribute throughout the cytoplasm as well as localize into the nuclear region of the cells (Alonso et al., 2010). When expressing the PH-tau in these cells, positive staining for caspase 3 and TUNEL was detected suggesting that the cells are in a state of apoptosis (Alonso et al., 2010). Moreover, the presence of the R406W mutation seemed to cause an increase in the level of caspase activation supporting the idea that this mutation makes the Tau protein more prone to becoming a toxic molecule.

Taken together, this data supports the finding that specific sites of phosphorylation, when together, drive the protein to become toxic, rather than just the sheer amount of phosphate associated with the protein. The behavior of the PH-tau raised questions as to what specifically was happening in the cells. One such question was the reduction of size and rounding up of the cells, which lead to beginning to question the stability of the actin microfilaments. The work presented in this study began to explore

the cellular pathology seen when PH-tau is expressed in cell culture, specifically the stability of the actin cytoskeletal components.

Actin microfilaments are a highly conserved component of the cytoskeleton found within all eukaryotic cells (Aylett et al., 2011). Their arrangement is two strands of polymers wound together ranging from 5-9nm in width. They can be found in several configurations in the cell but are predominantly found in the soma most closely associated with the plasma membrane (Schoenenberger et al., 2011). Like microtubules, these structures are also dynamic, in that they can grow and breakdown as needed by the cell, but this dynamic process require the utilization of nucleotide hydrolysis (Aylett et al., 2011).

MAP4 binds to actin at its microtubule binding domain (Matsushima et al., 2012). This was done using bacterially expressed MAP4 and its truncated form *in vitro*. Matsushima also reports that under electron microscopy, actin filaments that were associated with MAP4 were longer and straighter than those without MAP. Actin and tau have been shown to interact at tau's proline rich region as well as the microtubule binding domain (He et al., 2009). This was shown using truncated and deletion tau mutants. Furthermore, He and colleagues also found that tau was able to interact with F-actin and promote F-actin formation. Sharma and colleagues (2007) have shown that tau interferes with growth-factor mediated actin remodeling. Cells where the actin stress fibers were broken down usually recovered fibers in 5-7 hours, where those cells expressing tau, had a far more prolonged recovery period (Sharma et al., 2007).

Actin does play a role in neurodegeneration. There is evidence that cofilin, a primary molecule involved in actin dynamics, is associated with MAP tau when it is

aggregated inside the cell (Whiteman et al., 2009). With the breakdown of the microtubules and the entrapement of cofilin in the neurofibiriallary tangles, these may tie in to neurodegeneration not exclusively from microtubule instability, but also from the instability of the microfilament components.

It has been shown that microfilaments play a major role in the formation and regulation of synaptic boutons (Bleckert et al., 2012). Alzheimer's disease causes the loss of synaptic terminals and the retraction of neurites (Pozueta et al., 2012). The breakdown of synapses between neurons may not be exclusively due to a destabilization of the microtubule network. The role that actin plays in synapse regulation may be a connection to Alzheimer's disease causing the synaptic boutons to be lost.

Results presented here suggest that the actin cytoskeleton is also destabilized as a result of tau hyperphosphorylation. This work looks at the structure of actin in CHO cells expressing PH-tau. Through collaboration with the CUNY CSI Computer Science Department, a new plugin for ImageJ has been developed to quantify the amount of actin present in cells in relation to the amount of tau that is being expressed.

3.2 - Materials & Methods

3.2.1 - Generation of GFP fused Pseudophosphorylated Tau Constructs & Plasmid Purification

PH-tau vectors were generated according to Alonso et al. (2010). The original pEGFP vector containing wild type tau was a gift from Dr. Michael Novak (Institute for Neuroimmunology, Bratislava, Slovak Republic). The GFP-fused pseudophsohprylated tau constructs were prepared specifically for this work in order to visualize tau in a live

cell imaging system. To do this, the pAcGFP-C1 vector (Clontech) were digested with BglII and EcoRI (Promega). The pseudophsphorylated tau sequence was cut with the same enzymes from the original, non-fluorescent vectors used in the previous work. These particular enzymes allowed us to insert tau in reading frame with the GFP gene and create a fusion protein. The pAcGFP vector was ligated with the tau gene using T4 ligase according to the manufacturer (Promega). The ligation mixture was then transformed into chemically competent *E.coli* (Fisher Scientific) and plated on Lauria Bertani plates (LB; 10g NaCl, 10g tryptone, 5g yeast extract in distilled water, autoclaved at 121°C at 15 psi for 15 minutes) which were supplemented with kanamycin (0.1mg/ml, from Fisher Scientific). Colonies were screened for the tau gene by restriction digest of purified plasmid and those cells positive glycerol stocked at -80°C. To purify plasmid DNA, freezer stocks were re-plated in LB plates supplemented with Kanamycin, incubated overnight at 37°C and a single colony was re-suspended in LB broth supplemented with kanamycin. The following day, plasmid was isolated using the Promega Wizard Plus SV DNA purification kit (Promega) according to the manufacturers protocol.

3.2.2 - Cell lines used and transfection

Chinese hamster ovarian fibroblast cells were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS, from Fisher Scientific) and 100 units of penicillin, 100μg streptomycin (Invitrogen, as a 100x solution). Cells were grown at 37°C in the presence of 5% CO_2. The day before transfection, cells were plated to 70-

80% confluency. The plating vessel was determined by the type of experiment and will be mentioned in each section.

Cells were transfected with the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. Generally, cells were maintained for 48 hours post fixation prior to the start of any imaging or further processing.

3.2.3 - F-Actin Staining

Cells were plated in 8 well chambered slides (Lab Tek II) and transfected the following day with the appropriate plasmid according to the procedure above. Forty-eight hours post transfection, the cells were fixed with 4% paraformaldehyde at least overnight. The actin stress fibers in the cells were labeled with rhodamine-phalloidin (Cytoskeleton Inc.) according to the manufacturers protocol. Briefly, the cells were washed with three times five minutes each with PBS, incubated for one hour at room temperature in rhodamine-phalloidin which was diluted in PBS. The cells were washed again and then coverslipped with Vectorshield antifade mounting medium (Vector Laboratories Cat #H1000).

3.2.4 - Okadaic Acid treatment & Actin Labeling

CHO cells were plated in eight well chamber slides (Lab-Tek II) and transfected the following day with fluorescent wild type Tau (pAcGFP-Wt-tau). Twenty-four hours after transfection, the cells were treated with either 50nM or 100nM okadaic acid mixed in complete growth medium (Sigma-Aldrich Cat #O-8010) for another 24 hours. Cells were then fixed with 4% paraformaldehyde in PBS pH 7.2 overnight. The F-actin in the cells was stained according to the protocol above.

3.2.5 - Immunocytochemistry

CHO cells were plated in eight well chamber slides (Lab-Tek II) and transfected the following day with the fluorescent tau constructs. Forty eight hours post transfection, the cells were fixed with 4% paraformaldehyde in PBS pH 7.2 and immunocytochemistry was carried out as follows: the cells were washed with PBS, permeabilized with 0.1% Triton X-100 (Fisher Scientific Cat # BP151-100), treated with 0.1M glycine (Fisher Scientific) to quench aldehyde autoflorescence, blocked in 4% donkey serum in PBS with 0.01% tween 20 (PBST) and incubated overnight in primary antibody mouse anti Pan actin Ab-5 (Actn05) (Neomarkers Cat # Ms-1295-P) which binds to both polymerized and free actin in the cell. The following day, the cells were washed with PBS and incubated in PBST with donkey anti-mouse conjugated to Cy3 (Jackson Immunoresearch Cat #715-165-150) for one hour, washed again with PBS and coverslipped with Vectorshield antifade mounting medium (Vector Laboratories Cat #H1000). Cells were imaged using a Leica SP2 AOBS confocal laser scanning microscope. Total fluorescence was quantified using ImageJ. Statistical analysis was perfomed in Microsoft Excel 2007.

3.2.6 - Actin Intensity Measurements

In order to be able to quantify the total amount of actin in PH-tau and wild type expressing cells, the CUNY CSI Computer Science Department has begun to develop a new plugin for ImageJ that will analyze every pixel in an image and determine if that pixel contains green, red, both together or neither at all. The images are loaded though ImageJ and the plugin is started. For each group of images, a global threshold is set for

each of the colors and the total amount of green and red intensity (a total of all the pixels) is plotted using Microsoft Excel.

3.3 - RESULTS

3.3.1 - Rhodamine-Phalloidin staining in Tau expressing cells shows a breakdown of F-actin

Rhodamine-phalloidin is classically used stain F-actin in cells. In this application, phalloidin was used to bind to F-actin in the cells. F-actin was of particular interest because the change in size of the cells was believed to be related to a depolymerization of F-actin. When looking at wild type tau expressing cells, the F-actin network, commonly seen at the membrane surface, appears to be complete (Figure 3.1A). The filaments are long and easily comparable to surrounding cells, ones that are not expressing any foreign protein. These cells maintain their healthy CHO cell appearance.

When looking at cells that are expressing PH-tau, the F-actin appears to be broken down (Figure 1B-D). The staining in some cases appears very punctate (Figure 3.1B & C). These cells do have some polymerized F-actin at the immediate surface of the membrane, but there are no visible fibers that spread out through the center for the cell body. Additionally, there is a total lack of F-actin staining at all (Figure 3.1D). These cells appear as a void in the confluent cell surface. It is also notable that PH-tau enters the nucleus in PH-tau expressing cells. Taken together, these results suggest that PH-tau is causing a breakdown of the actin microfilaments.

3.3.2 - Intensity of actin presence in different tau transfected cells

A custom ImageJ plug-in is applied to each original cell image. The user enters parameters specifying thresholding values (minimum and maximum for each channel). In addition, a difference-threshold is entered. The parameter values for these results are: min and max for red (30,255) and green (30,255) with the difference threshold set to 30. The pixels in the image are processed using the values within the threshold limits. In computing the channel area ratios, values whose differences are above the threshold are considered. The area ratios are displayed as well as the cumulative values (here the numbers are weighted by the intensity values). Finally a visualization of the included areas is drawn. The red and green areas denote the thresholded pixels while the white indicates indeterminate values.

This plugin was used to quantify the total amount of actin and tau present in these cells (Figure 3.2). A threshold was set for each set of cells. The amount of wild type tau expressed in a cell does not change the level of F-actin in the cells. This is seen when comparing the levels of red (y-axis, actin) to the level of green (x-axis, tau). There is no decrease in the amount of red as the level of green increases. Therefore, no matter how much wild type tau the cell is producing, the level of actin present does not change.

The PH-tau samples however show that there is a decrease in red, or actin staining as the level of tau expression increases in the cells. Actually, the level of tau is inversely proportional to the level actin, or the higher the signal of tau, the lower the signal there is from actin. The presence of a best-fit line on each graph demonstrates this relationship.

3.3.3 - Immunocytochemical Staining of Actin does not show any differences between wild type and PH-tau

In order to see if there was a change in the level of total actin in the PH-tau and wild type tau expressing cells, total actin immunocytochemistry was used (Figure 3.3). In Figure 3.3, panels A, WT-tau and B, PH-tau, visually, there appears to be no difference in the total level of actin in the cells. This leads one to believe that the disruption that is being seen does not have to do with the expression of actin or exclusively on the entrapment of actin in the aggregated tau, but there may be some interaction of tau directly with actin, or with one of the actin polymerizing proteins.

To better quantify the amount of total actin within the wild type tau and PH-tau expressing cells, ImageJ was used to calculate the fluorescence intensity. This revealed that there is no statistical difference between the PH-tau and WT-tau total actin levels.

3.3.4 - Okadaic Acid treated wild type transfected cells causes the same F-actin breakdown

Due to the use of the pseudophosphorylated tau constructs, the ability to see if actual phosphorylation drives the same pathology is important. To accomplish this, cells transfected with fluorescent wild type tau were treated with 100nM okadaic acid for 3 hours prior to fixation. This toxin blocks the function of the phosphatases in the cells. With these enzymes blocked, it has been shown that wild type tau does become hyperphosphorylated and that of the sites that are phosphorylated, T212, T231 and S262 are all included.

Figure 3.4 demonstrates the results seen in the Okadaic acid treated cells. In panels A and B, the fragmented and punctate staining of F-actin can be seen. The actin

fibers of the surrounding cells, those not expressing tau, appear to have a healthy network of F-actin. This supports that idea that at this concentration for that period of 3 hour, the Okadaic acid was not toxic to the cells, but rather the hyperphosphorylation of tau drove the breakdown of the actin filaments.

3.4 - DISCUSSION

The actin cytoskeleton plays a major role in the structure of a cell by providing a great deal of support at the inner surface of the plasma membrane. It has been demonstrated that F-actin directly interacts with NESH, a protein that is part of the Abi gene family that regulates cell motility. This protein is expressed predominantly in the brain, and has been shown to play a role in new synapse formation (Bae et al., 2012b). It has been shown that NESH levels in synaptic buotons is regulated by association with F-actin. It has been seen that the disruption of F-actin in leads to a re-distribution of NESH out of the synapses and it is believed that this may play a role in the synaptic plasticity (Bae et al., 2012a). This study has shown that hyperphosphorylated tau appears to cause a depolymerization of the F-actin. It has been shown in former works that hyperphosphorylated tau also causes a retraction of neurites (Dubey et al., 2008). Without the presence of F-actin in these PH-tau cells, the change in cellular distribution on NESH may be a possible mechanism of tau neurodegeneration. At this time, there is no work looking into the relationship with NESH and MAP tau.

Cofilin, a major actin depolymerization molecule found in the central nervous system, has been shown to be responsible for the production of actin cofilin rods in Alzheimer's disease and that these rods also associate themselves with the microtubule

binding domain on MAPs (Whiteman et al., 2011)). Furthermore, the formation of actin cofilin rods plays a role in dendrite microtubule destabilization and contributes to compromised cellular transport, which contribute to synaptic loss (Cichon et al., 2012). The present study does not look for the presence of actin cofilin rods, just actin stress fibers. It has been shown that phalloidin staining does not label actin cofilin rods (Nishida et al., 1987). Since it has been shown that these structures can lead to neurodegeneration, future work will look into the presence of actin cofilin rods within PH-tau expressing cells which appear to be void of polymerized actin, but posses what appears to be a normal healthy amount of G-actin. The design of the present study is limited to analyze this question because CHO cells are not of neuronal origin and therefore will not express cofilin. To begin to answer this question, the lab's new transgenic mouse line expressing the same PH-tau used in this study, will be used to investigate the actin cofilin rods both *in vivo* and in primary culture models.

Additional work has shown that actin plays a role in exocytosis regulation. In a recent study by Wang and Richards (2011), they have shown that a disruption of the actin cytoskeleton causes a decrease in vesicle movement (Wang and Richards, 2011). In our previous work on membrane blebbing, tied with actin stress fiber loss, there may be a correlation between the lose of actin and the uncontrolled membrane blebbing is actin does in fact play a role in exocytosis regulation. It has recently been demonstrated that actin plays a major role in the coating of exocytotic vesicles and regulates the process as a whole (Nightingale et al., 2012). Without the presence of dynamic actin, the release of exocytotic blebs could occur uncontrollably, which is what we have seen in our earlier

work examining excessive membrane zeiosis. The zeiotic processes may be a result of the loss of dynamic F-actin in the presence of PH-tau.

The use of okadaic acid treatment tested of the ability of actual hyperphosphorylated tau to cause the same actin disruption. For this, wild type tau was transfected into cells and exposed to 100nM okadaic acid for three hours prior to fixation. These cells exhibited the same breakdown and punctate appearance of actin in the cytoplasm of the cells. This suggests that the pseudophosphorylation model is causes a similar change in the tau protein to cause the same changes in cellular pathology, first seen in membrane blebbing and now seen when analyzing the actin cytoskeleton distribution.

The development of the new ImageJ plugin to quantify the level of actin in tau expressing cells demonstrated that the level of actin is usually inversely proportional to the level of tau being expressed in the cells. The plugin worked by analyzing each pixel of an individual cell once cut out form the original captured photomicrograph. The plugin was able to determine if the pixel was red, for actin, green, for tau or a mixture of the two. First, using this program, it was easily determined at which regions of the cell that both actin and tau were in the same area when we were able to see which pixels contained both colors. The plugin will be further configured with a special focus on adjusting the threshold. A future goal would be to be able to get a threshold that would be satisfactory for all. This will need some more configuration and further imaging on the microscope in order to be able to have a large pool of images captured with different parameters to see which images are best and what makes for a solid threshold level.

Further work looking into the linearity of the actin fibers is also being explored. This will allow for he measurement of the actin stress fibers present in the cells under different conditions. Future data will analyze the length and number of these fibers. Together, these plugins will be useful to any project studying actin distributions within cells. Additionally, primary neuronal cultures will be prepared and transfected with the wild type and PH-tau constructs to analyze the synapse formation and maintenance as well as axonal stability in a neuronal model. The level of actin will be analyzed in our transgenic mouse model as well.

3.5 - CONCLUSION

This study makes a novel observation that the F-actin stress fibers are not intact in PH-tau expressing cells. Even though the F-actin is not detected, using total actin labeling through immunocytochemistry, the cells seem to express G-actin at the same levels regardless of PH or wild type tau expression.

Okadaic acid treated wild type tau expressing cells seemed to exhibit much the same actin pathology as those cells expressing the PH-tau. This suggests that hyperphosphorylation of tau does lead to the breakdown of the actin cytoskeleton and that the phosphorylation sites T212, T231 and S262 play a major role in the progression of tau induced neurodegeneration.

In this work, it was seen that the level of F-actin expression is inversely proportional to the level of PH-tau present in the cells. Wild type tau did not seem to cause a major depolymerization in the appearance of stress fibers.

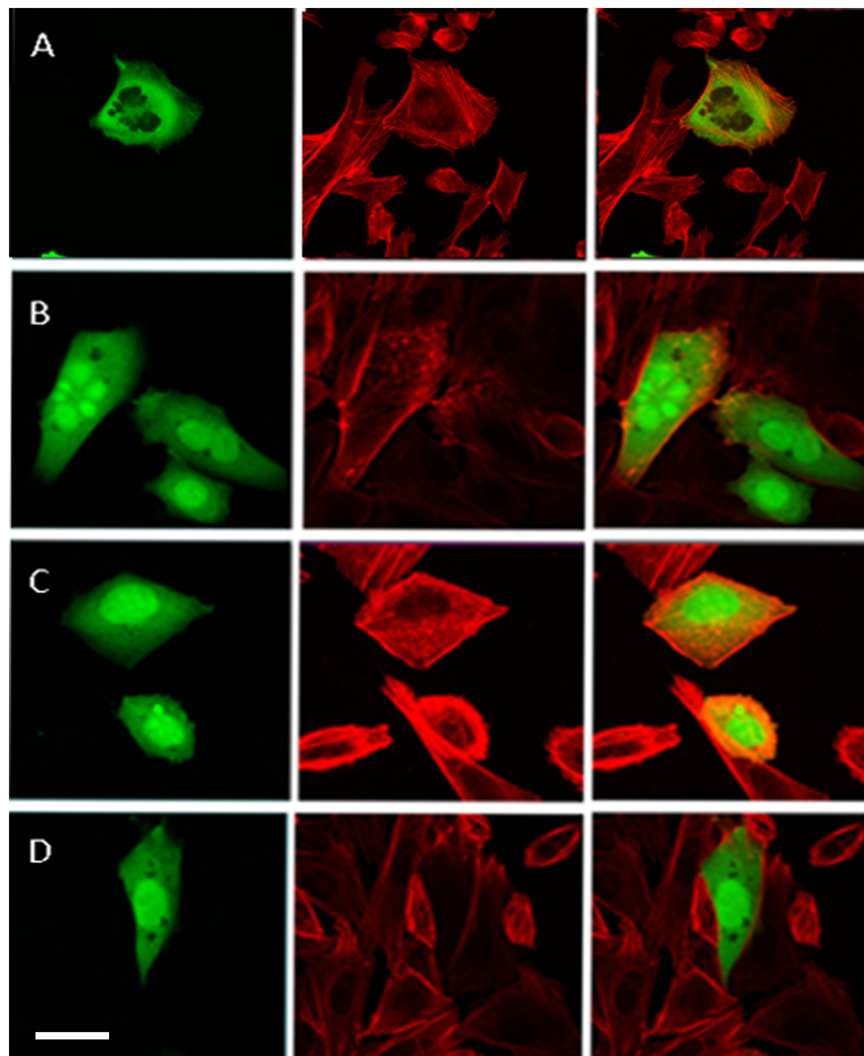


Figure 3.1 – F-actin polymerization is decreased in PH tau expressing cells - CHO Cells were transfected with either fluorescent wild type tau (A) or PH-tau (B-D). The cell expressing wild type tau have normal F-actin present in the cell body. It can also be seen that wild type tau does not enter the nucleus of the cell. The F-actin network in cells expressing PH-tau, it is seen that the filament are disrupted. In some cases (B & C), the actin is no longer filamentous, but is punctate throughout the cytoplasm. It can also be seen that actin is not at all present in other PH-tau expressing cells (D). Scale bar represents 15µm.

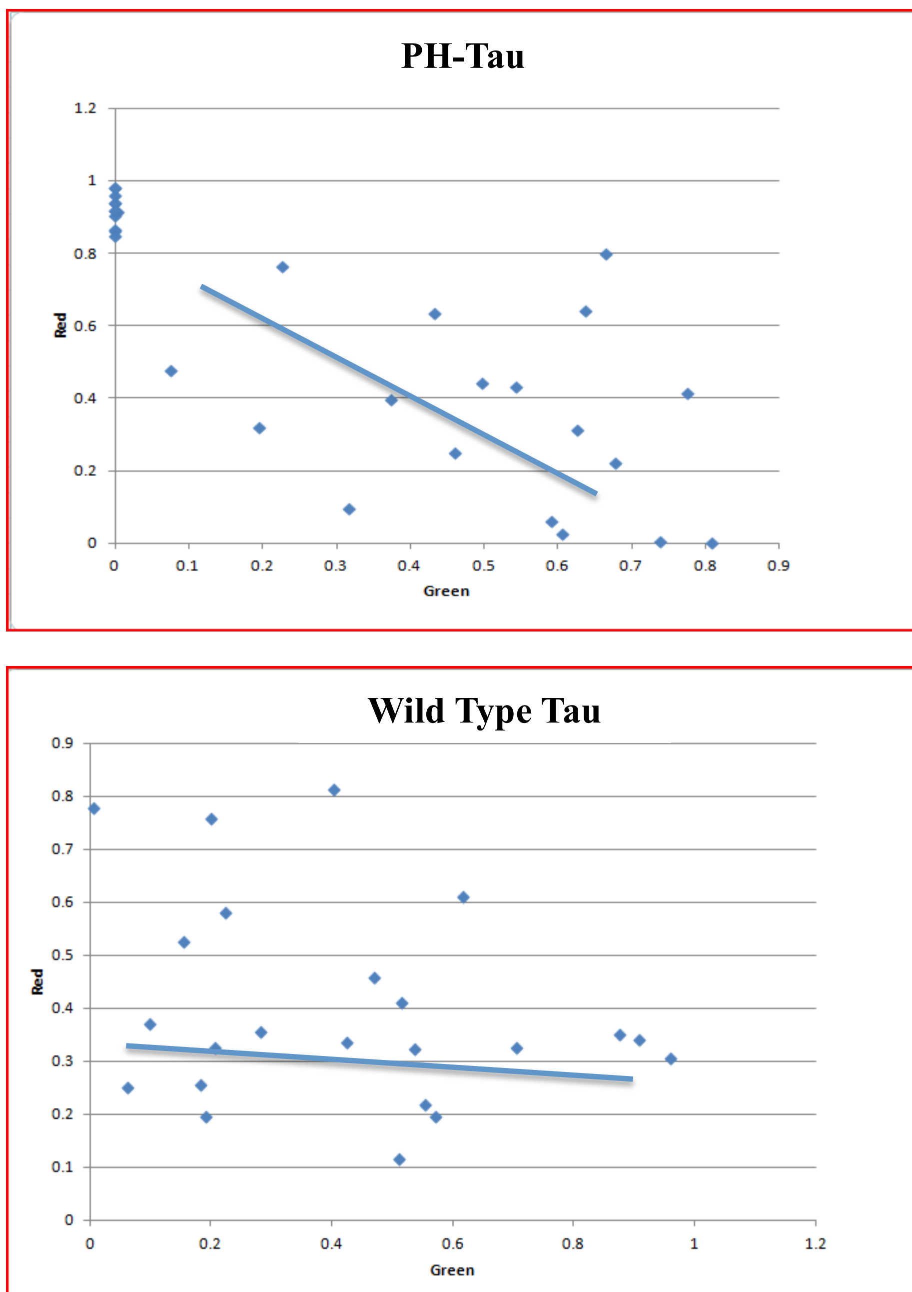


Figure 3.2 – Quantification of F-actin and Tau levels in Wild type and PH-tau expressing cells – To quantify the amount of F-actin and tau in transfected CHO cells, an ImageJ plugin is being developed. This plugin was utilized on 24 wild type and PH-tau expressing cells and the levels of the actin and tau were plotted (y axis and x axis respectively). When looking at the trend in the cells, the level of wild type tau (green) has no effect on the level of F-actin expressed in the cells. However, in PH-tau cells, the higher the level of tau, the less F-actin is detectable. This correlated to what we have seen in the images like those presented in Figure 1.

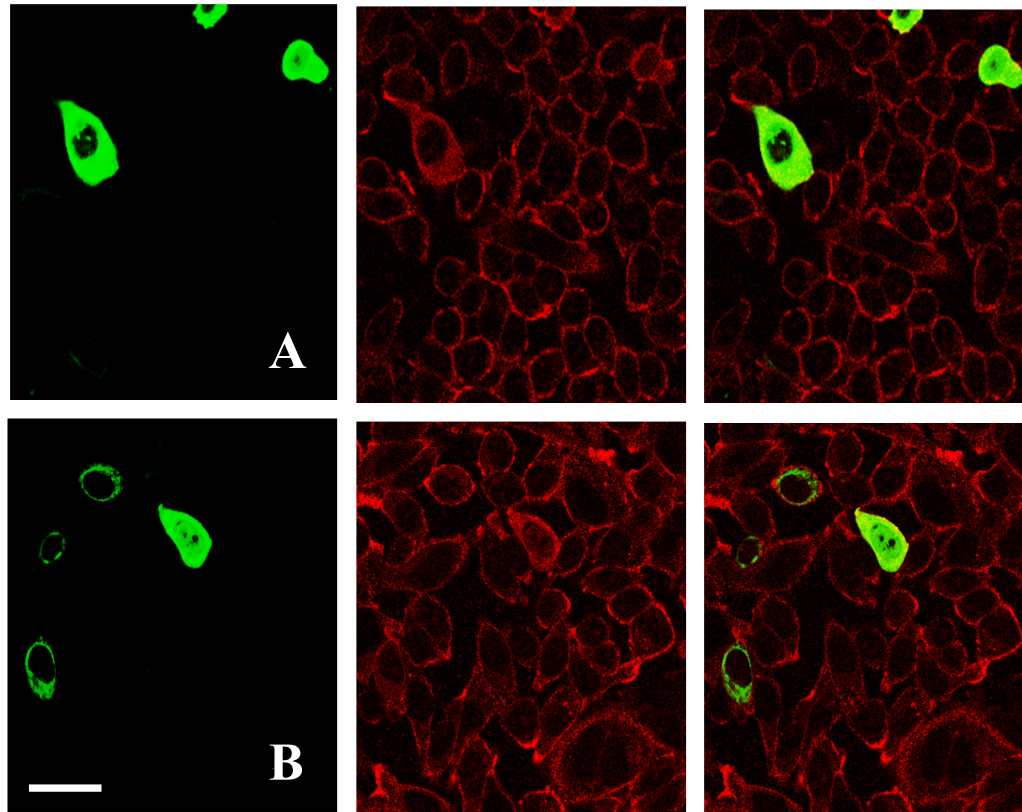


Figure 3.3 – Levels of G-actin are not changed – CHO Cells were transfected with either fluorescent wild type or fluorescent PH-tau and fixed for immunocytochemistry 48 hours later. Antibody labeling of total actin was done to compare with the rhodamine-phalloidin labeling of F-actin. Statistically, there is no differencec between The amount of total actin PH-tau expressing cells (B) when compared to wild type (A), only the F-actin, or polymerized form is interrupted. Scale bar represents 20µm

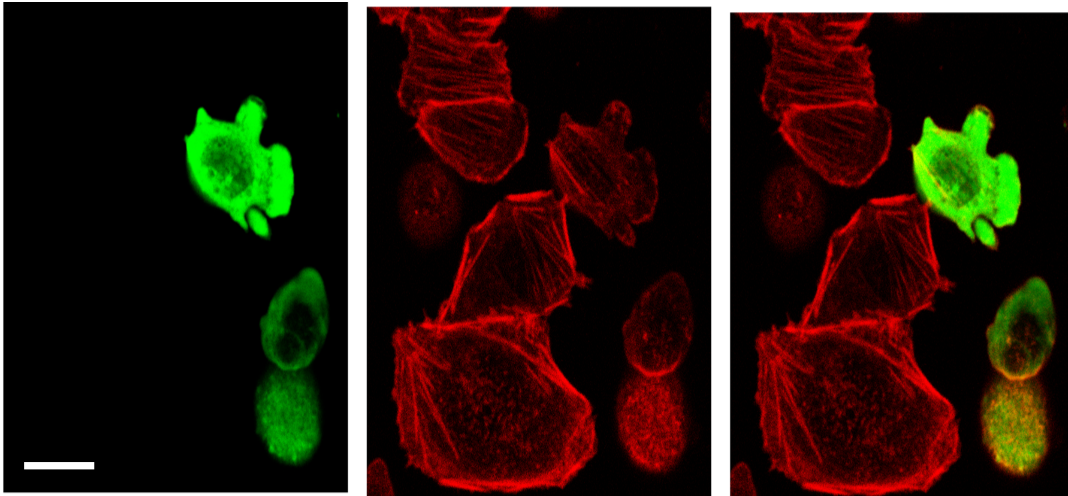


Figure 3.4 –F-actin labeling in Okadaic Acid Treated cells expressing Wild type tau: These cells are transfected with wild type tau and treated with 100nM Okadaic Acid for 3 hours prior to fixation. This treatment blocks the function of phosphotases and therefore causes wild type tau to be hyperphosphorylated. The actin cytoskeleton is also compromised in these cells. The actin appears to be very punctate in its appearance. There is also the lack of F-actin stress fiber present. Scale bar represents 10μm.

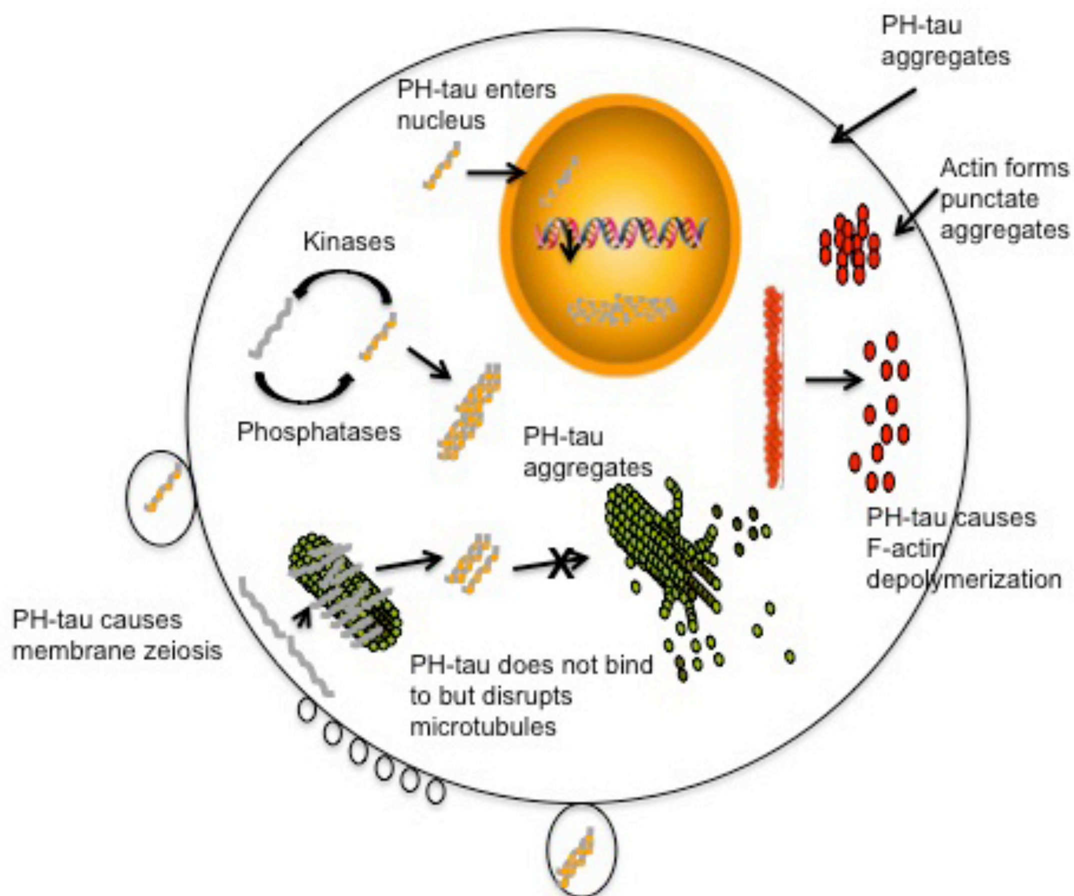


Figure 3.5 – Summary Drawing of cellular events taking place when PH-tau is expressed in cultured cells – This summary diagram outlines the depolymerization of F-actin in PH-tau expressing cells. Also, depicted is microtubule disruption and the presence of PH-tau in the nucleus.

CHAPTER FOUR

Elimination of an importin binding site on PH-tau inhibits entry into nucleus

Abstract: Hyperphosphorylation of the microtubule associated protein tau is involved in several dementias that classify as tauopathies. In these diseases, tau is found hyperphosphorylated and is known to bind to itself rather than associate with microtubules. Without cytoskeletal support, neurons begin to loose their ability to function and therefore there is a loss of neuronal arborization. When chinese hamster ovarian cells (CHO) are transfected with PH-tau (full length human tau pseudophsohproylated at T212, T231 and S262) in addition to not associating with microtubules, this protein can be found in the nucleus of the cells. The presence of this protein in the nucleus could be contributing to the induced neurodegeneration seen in tauopathies. We found a nuclear localization signal that allows the chaperon protein importin to bind to tau. To see if this site was responsible for the translocation of tau into the nucleus, we eliminated importin's binding site through site directed mutagenesis of the full length tau gene. The elimination of the importin binding site inhibited tau from being able to translocate into the nucleus. The cells expressing PH-tau without the importin binding site did appear healthier than those expressing regular PH-tau. They cells were less rounded up and the microtubule network seemed to be overall in a healthier state. However, the lack of tau in the nucleus did not prevent the breakdown of the microfilaments and did not change the level of caspase 3 activation in the cells and in the culture as a whole. These results suggest that the cytoplasmic hyperphosphorylated tau is the toxic molecule that induces disruption of the actin cytoskeleton and causes the caspase 3 activation. Further investigation will be needed to determine the role if any of the hyperphosphorylated tau nuclear translocation in tau-induced neurodegeneration

Keywords: tau, tauopathies, Alzheimer's disease, neurodegeneration, importin(s)

4.1 - INTRODUCTION

Tau is the microtubule-associated protein (MAP) that is specific to neuronal cells. Tau is the primary MAP that provides stability to the microtubules within the axons. These filaments are responsible for both structural support as well as intracellular and intra-axonal transport. Most of the necessary cellular components, such as packaged neurotransmistters and mitochondria, required at the axon terminal are created and packaged in the cell soma and are relocatized to the axon terminal. The stabilized microtubule network is used as the “highway” by which all of these molecules are moved from the soma to the axonal terminal via the movement of the motor proteins, such as dyanin and kinesin (Cho and Vale, 2012; Hirokawa, 2011). Without this cellular movement within neuronal cells, the axonal structure would be compromised and the lack of microtubule tracks, ability to propagate signals will also be disrupted. This will eventually lead to cell death.

Tauopathies, which include Pick’s disease and Alzheimer’s disease, are classified as a group of neurogenerative diseases that are caused by the dysfunction of MAP tau. In above mentioned neurodegenerative disorders, tau no longer binds to microtubules, but is hyperphosphorylated and aggregates into paired helical and straight filaments and is found in a hyperphosphorylated state (Alenghat et al., 2008; Alonso et al., 2001b; Cho and Vale, 2012; Goedert et al., 1992; Gomez-Ramos et al., 2004; Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Hirokawa, 2011; Iqbal et al., 1986). These filaments join together to form the neurofibrillary tangles, the classically seen intracellular protein aggregate characteristic to Alzheimer’s disease. With the lose of the stabilized microtubules, in addition to cell deterioration, there is now no further axonal transport,

which leads to compromised neuronal signaling and leads to a retraction of neurites and ultimately cell death.

There are six tau isoforms seen in humans and are generated by alternative splicing of the pre-mRNA. These isoforms can include three or four microtubule binding domains, all which reside at the C-terminal domain as well as zero, one or two 29 amino acid N-terminal inserts (Goedert et al., 1989; Himmler et al., 1989).

There are several post translational modifications that occur on tau. Phosphorylation is one such modification that, we propose, play a role in tau's switch to a pathologic protein. At normal phosphorylation states, there is approximately 3 moles of phosphate per molecule of protein. In this hyperphosphorylated state, the protein carries around 8 moles of phosphate per molecule of protein (Köpke et al., 1993). The sites that are most seen phosphorylated when tau is hyperphosphorylated are Thr212, Ser214, Thr231, Ser235, and Ser262 (Brandt et al., 1994; Drewes et al., 1995; Jenkins and Johnson, 1997; Sengupta et al., 1997; Singh et al., 1996). Additionally, these sites were shown to be involved in tau self assembly (Alonso et al., 2004).

Previous work has shown *in vitro* that hyperphosphorylated tau induced self-assembly and the formation of filaments as well as the inability to promote microtubule assembly (Alonso et al., 2001c). It has also been shown that phosphorylation at Ser262 accelerates tau assembly into filaments.

The discoveries of mutations on the tau gene have demonstrated that tau mutations alone are enough to promote dementia in patients (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). There are three reported types of tau mutations; missense, deletion and intronic. Previous work has shown that *in vitro* the FTDP-17

mutations have given tau the ability to self-assemble when containing approximately half the level of phosphate molecules when compared to wild type tau (Alonso et al., 2004). When tau is phosphorylated, we proposed that there is a conformational change that makes it a pathogenic protein and that this conformational change is more easily reached if the protein carries the FTDP-17 mutation (Alonso et al., 2004).

Our earlier work has looked into the effect that specific sites of phosphorylation has on the tau protein. In order to mimic site-specific phosphorylation, specific sites were pseudophosphorylated. To do this, certain codons were changed by site directed mutagenesis, which altered amino acids in the protein. Specifically, tau had specific serine and threonine residues switched with glutamic acid. This amino acid switch mimicked the negative charge that the addition of a phosphate group at that site would cause.

In 2010, six phosphorylation sites were analyzed (S199, T212, T231, S235, S262 and S396) (Alonso et al., 2010). When each single site was pseudophosphorylated, none were able to cause tau to disassociate with microtubules, with or without the FTDP-17 mutation (R406W). Interestingly, S262 did cause tau to associate with microtubules more weakly (Alonso et al., 2010). Two sites pseudophosphorylated in conjunction also did not cause tau to stop associating with microtubules. It was only when, specifically, T212, T231 and S262 were pseudophosrylated both with and without the FTDP-17 mutation, R406W did tau stop binding microtubules, disrupted the preformed ones and induced caspase 3 activation and positive TUNEL staining.

When T212, T231 and S262 were pseudophosphorylated, tau stopped binding to microtubules. We refer to this tau as pathological human tau (PH-tau; full length tau

pseudophosphorylated at T212, T231 and S262). Tau, was found distributed throughout the cytoplasm as well as localized into the nucleus of the cells (Alonso et al., 2010). The presence of the R406W mutation seemed to cause an increase in cell numbers that were positive for caspase activation. This supports that the FTDP-17 mutations make tau more toxic.

Taken together, this data supports the finding that specific sites of phosphorylation, when together, drive the protein to become toxic, rather than just the sheer amount of phosphate associated with the protein.

Certain chaperone proteins are responsible for tagging and assisting other proteins for entrance into the nucleus. The cell nucleus is a rather protected region within the cell and entry to just any molecule is not permitted. It is required that these chaperone proteins assist in the process. The karyopherins, which contain the importins and the exportins, are such chaperone proteins (Lott and Cingolani, 2011). Importins are responsible to mark cytoplasmic proteins for movement from the cytoplasm into the nucleus through the nuclear pore complex while exportins work in exactly the opposite manner, marking nuclear componets, including mRNA for movement into the cytoplasm through the nuclear pore complex (Chu et al., 2007).

The movement of these proteins occurs by the binding of these karyopherin proteins to the nuclear localization signal (NLS) on the protein to be translocated. Our group found that the importin nuclear localization signal KKXK in the tau sequence at amino acid residues 140-143. Previous work has shown that cells expressing PH-tau had a large volume of tau in the nucleus.

The elimination of the importin binding site on tau was able to inhibit the PH-tau from translocation into the nucleus. The microtubule network seems to be more stable and the cells seem to maintain a healthy morphology. Even with the visibly healthier cells, the F-actin polymerization was still affected and the increased level of caspase 3 activation was not changed.

4.2 - MATERIALS & METHODS

4.2.1 - Generation of GFP fused Pseudophosphorylated Tau Constructs & Plasmid Purification

Non-fluorescent PH-tau vectors were created for a previous study according to Alonso *et al.* (2010). The original pEGFP vector containing wild type tau was a gift from M. Novak. The GFP-fused pseudophsohprylated tau constructs were prepared specifically for this work in order to visualize tau in a live cell imaging system. To do this, the pAcGFP-C1 vector (Clontech) was restricted with BglII and EcoRI (Promega). The pseudophsphorylated tau sequence was restricted with the same enzymes from the original, non-fluorescent vectors used in the previous work. These particular enzymes allowed us to insert tau in reading frame with the GFP gene and create a fusion protein. The pAcGFP vector was ligated with the tau gene using T4 ligase according to the manufacturer (Promega). The ligation mixture was then heat shock transformed into chemically competent *E.coli* (Fisher Scientific) and plated on Lauria Bertani plates (LB; 10g NaCl, 10g tryptone, 5g yeast extract in distilled water, autoclaved at 121°C at 15 psi for 15 minutes) which were supplemented with kanamycin (0.1mg/ml, from Fisher Scientific). Colonies were screened for the tau gene by restriction digest of purified

plasmid and those cells positive glycerol stocked at -80°C. To purify plasmid DNA, freezer stocks were re-plated in LB plates supplemented with Kanamycin, incubated overnight at 37°C and a single colony was re-suspended in LB broth supplemented with kanamycin. The following day, plasmid was isolated using the Promega Wizard Plus SV DNA purification kit (Promega) according to the manufacturers protocol.

4.2.2 - Cell lines used and transfection

Chinese hamster ovarian fibroblast cells were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS, from Fisher Scientific) and 100 units of penicillin, 100 μg streptomycin (Invitrogen, as a 100x solution). Cells were grown at 37°C in the presence of 5% CO_2. The day before transfection, cells were plated to 70-80% confluency. The plating vessel was determined by the type of experiment and will be mentioned in each section.

Cells were transfected with the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. Generally, cells were maintained for 48 hours post fixation prior to the start of any imaging or further processing.

4.2.3 - Elimination of the importin binding site by site directed mutagenesis

Using the non-fluorescent PH-tau and wild type tau constructs, site directed mutagenesis was used to alter the specific codons to change the to amino acids in the putative importin binding site on tau. Two sites on tau were altered, K140A and K141A (Figure 4.1). Site directed mutagenesis was performed using the Stratagene Quickchange Lightening. Positive transformants were analyzed by DNA sequencing to determine is the plasmid was carrying the K140A/K141A mutations. To prepare the GFP-fused tau with

the AAAK mutation, the non-fluorescent tau was cloned into the pAcGFP-C1 vector using the same restriction enzymes to prepare the original GFP-fused tau vectors.

4.2.4 - Immunocytochemistry

CHO cells were plated in eight well chamber slides (Lab-Tek II) and transfected the following day with the fluorescent tau constructs. Forty eight hours post transfection, the cells were fixed with either 4% paraformaldehyde PBS pH 7.2 or with 100% methanol (for permeabilization) in and immunocytochemistry was carried out as follows: the cells were washed with PBS, permeabilized with 0.1% Triton X-100 (Fisher Scientific Cat # BP151-100), treated with 0.1M glycine (Fisher Scientific) to quench aldehyde autoflorescence, blocked in 4% donkey serum in PBS with 0.01% tween 20 (PBST) and incubated overnight in primary antibody. The following day, the cells were washed with PBS and incubated in PBST with donkey anti-mouse conjugated to Cy3 or FLUOR (Jackson Immunoresearch Cat #715-165-150 or 715-486-020 respectively) for one hour, washed again with PBS and coverslipped with Vectorshield antifade mounting medium (Vector Laboratories Cat #H1000). Cells were imaged using a Leica SP2 AOBS confocal laser scanning microscope. Images were analyzed using ImageJ. The primary antibodies used were mouse anti tau DA9 for total tau (a gift from Dr. Peter Davies) and mouse anti DM1A which binds to the tubulin alpha subunit (Neo Markers Cat #MS-581-P).

4.2.5 - Determination of Caspase3 Activation

Cells were plated into 8- well chamber slides (Lab-Tek II) and each well was transfected with the appropriate GFP-fused tau vector. 48 hours post transfection, cells

were fixed with 4% paraformaldehyde overnight and were imunostained as described above using rabbit anti active caspase 3 (Millipore Cat #AB3623) as the primary antibody and Alexa Cy3 (Jackson Immunoresearch Cat #715-545-150) as the secondary antibody.

The slides were imaged on a Nikon Eclipse Ni-E equipped with Stereoinvestigator (MBF Bioscience). The system was used to collect the two different channels; GFP-fused tau, Cy3-caspase3. These images were opened in ImageJ and the cells labeled with the different colors were counted. Numerical data was tabulated and analyzed using Microsoft Excel.

4.2.6 - F-Actin Staining

Cells were plated in 8 well chambered slides (Lab Tek II) and transfected the following day with the appropriate plasmid according to the procedure above. Forty-eight hours post transfection, the cells were fixed with 4% paraformaldehyde at least overnight. The actin stress fibers in the cells were labeled with rhodamine-phalloidin (Cytoskeleton Inc.) according to the manufacturers protocol. Briefly, the cells were washed with three times five minutes each with PBS, incubated for one hour at room temperature in rhodamine-phalloidin, which was diluted in PBS. The cells were washed again and then coverslipped with Vectorshield antifade mounting medium (Vector Laboratories Cat #H1000).

4.3 - RESULTS

4.3.1 - Mutation of tau's importin binding site keeps tau out of the nucleus

PH-tau, when expressed in CHO cells, seemed to be present in large quantities in the cell nucleus and we hypothesized that this presence, being abnormal, could contribute to the toxic effect that PH-tau has on cells. We discovered that there was a nuclear localization signal on the tau protein that bound importin. Using site directed mutagenesis, we changed the codon for lysine into alanine (A) to eliminate the ability of importin to bind tau, yielding PH-tau-K140A/K141A.

The elimination of this importin binding site does keep tau from entering the cell nucleus. Figure 4.2 shows the fluorescent (A and B) PH-tau expressed in CHO cells. The PH-tau expressing cells (A) have tau present in the nucleus. In these cells, the appearance of membrane blebs apparent. In the PH-tau-K140A/K141A expressing the mutated importin site, tau is almost totally kept out of the nucleus (B). The microtubule structure seems to be more extensive in the larger, PH-tau-K140A/K141A cells. In panel B, it can be seen that tau is still not well associated with microtubules. In order to further analyze tau's ability to bind to microtubules, cells will be permeabilized prior to fixation to remove unbound protein. Additionally, *in vitro* microtubule binding assays can be done.

The ability of the microtubules to be present could be due to additional support from the native MAPs of the CHO cells, which are still being expressed. Further work will have to be done to look into the ability of both PH-tau and PH-tau-K140A/K141A to sequester those native MAP proteins.

4.3.2 - Microfilaments are still disrupted after eliminating tau's importin binding site

We have shown that the actin cytoskeletal components are broken down when PH-tau is expressed in cells. To see if the presence of tau in the nucleus played a role in

the microfilament breakdown, GFP fused PH-tau and PH-tau-K140A/K141A were transfected into CHO cells and the F-actin was labeled using rhodamine-phalloidin. Figure 4.3 shows the F-actin label in cells expressing both the PH-tau (A) and the PH-tau-K140A/K141A constructs. As before, tau is inhibited from entering the nucleus in the PH-tau-K140A/K141A expressing cells. However, the lack of PH-tau in the nucleus does not seem to improve the microfilament network. As was seen in the regular cells, the actin does not form stress fibers, but rather the cells contain punctate structures that labels as actin. These punctate structures are similar to those seen in an earlier study and have been seen again in PH-tau expressing cells. The presence of tau in the nucleus does not influence the overall polymerization of the actin cytoskeletal components.

4.3.3 - Caspase 3 levels do not change when tau's importin binding site is eliminated

It has been shown that there is an increase in caspase 3 activation in PH-tau expressing cells (Alonso et al., 2010). Furthermore, it has also been seen that caspase 3 is increased in cells not expressing PH-tau directly, but is only in contact with PH-tau expressing cells. To see if inhibiting tau from entering the nucleus caused a decrease in caspase 3 activation, cells transfected with PH-tau (Figure 4.4A) and PH-tau-K140A/K141A (Figure 4.4B) were imunostained with anti active caspase 3. As was seen earlier, the elimination of the importin binding site (PH-tau-K140A/K141A, Figure 4.4B) did keep tau from entering the nucleus. However, preventing tau from entering the nucleus did not cause a decrease in caspase 3 activation in either PH-tau expressing cells or surrounding cells.

4.4 - DISCUSSION

Earlier work showed that specific phosphorylation sites allowed a certain amount of tau to enter the nucleus. The presence of tau in the nucleus became more evident when using the GFP-PH-tau samples. Further investigation showed that there was a putative binding site for importin starting at amino acid 140 in the tau protein. This work demonstrates that the elimination of this binding site dose keep PH-tau out of the nucleus. Figure 4.5 is a summary diagram of the findings presented in this work. The cells expressing the PH-tau-K140A/K141A initially seem to be more spread out and not having the rounded up, dying appearance often seen in PH-tau expressing cells. The microtubule network was more expansive and the cells were larger in size. Membrane blebbing was detected in the cells expressing the PH-tau-K140A/K141A, which is often seen in PH-tau expressing cells. The presence of tau in the nucleus did play a part in driving the detected cellular pathology.

It was shown that although tau was kept out of the nucleus, there was no change in actin polymerization and caspase 3 activation stayed the same. The analysis of both caspase activity and F-actin polymerization did not show a morphological difference between PH-tau and PH-tau-K140A/K141A. This suggests that there is no correlation between the presence of PH tau in the nucleus and the activation of caspase 3. Also, PH-tau's presence in the nucleus does not trigger of the breakdown of the polymerized F-actin within the cells.

It has been shown by Hanz and colleagues that importins play a role in the regenerative process (Hanz et al., 2003). In axons, Importin beta was seen present far away from the cell soma and this importin is increased by the translation of local mRNA

at the sites of injury. The beta importin binds to any proteins containing a nuclear localization signal (NLS) and transports them retrograde when binding to dyanin. This interaction has been shown by this group to inhibit the regenerative process in these axons (Ben-Yaakov and Fainzilber, 2009; Hanz et al., 2003). The removal of the NLS from tau and preventing it from entering the nucleus does not stop F-actin depolymerization and the activation of caspase 3, but it seems to help the support the microtubule network. Their microtubule networks are healthier and the cells are less rounded. Further work will continue to investigate the mechanism by which importin plays a role in halting membrane zeiosis and helps with microtubule stability.

Additionally, it has been shown in Alzheimer's disease brain samples that there is an increase in importin alpha/1 in the Hirano bodies of the hippocampal neurons (Lee et al., 2006). This increase is believed to cause a dysfunction of the nuclear translocation signaling. This increase in specific importin molecules could account for the reason tau is taken into the nucleus so readily. Future work will look into specifically which importin proteins tau is able to bind in the CHO cell system. Additionally, the use of primary cultures from the labs inducible transgenic mice would be useful in seeing the role of importins in the axons and the role they play in neurodegeneration.

4.5 - CONCLUSION

It is clear that there is a nuclear localization signal on tau that is able to bind to importin when this protein in hyperphosphorylated. The elimination of this site does prevent tau from entering the nucleus of the cells and these cells. Additionally, keeping PH-tau out of the nucleus allows the cells to maintain a healthy morphology for a longer

period of time. This healthier state includes maintaining a healthier microtubule network and no membrane blebbing. However, the keeping PH-tau out of the nucleus does not stop the activation of caspase 3 in tau expressing and surrounding cells in culture nor does it stop the breakdown of the actin microfilaments.

More investigation will be required to analyze how PH-tau's presence in the nucleus adversely affects the stability of the membrane and the microtubule networks. Additionally, investigation into the specific importins that are involved in this process both in the CHO cell model as well as in primary cultures from our laboratory's inducible PH-tau expressing mouse model. Being able to understand which importins may be overexpressed when PH-tau is present and to which importin PH-tau specifically binds would prove to be valuable information to further investigate PH-tau and its role in the nucleus.

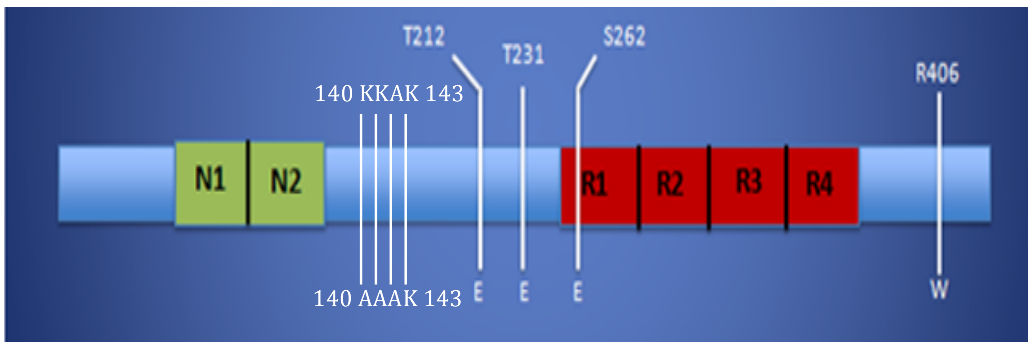


Figure 4.1 - Schematic of the full length human tau showing the location and generated mutation of the putative importin binding site - To eliminate the putative importin binding site 140 KKAK 143, two lysine (K) residues were switched to alanine (A) by site directed mutagenesis yeilding the sequence 140 AAAK 143. The other alterations are the sites of pseudophosphorylation (T212E, T231E and S262E) as well as a mutation associated with frontal temporal dementia (R406W).

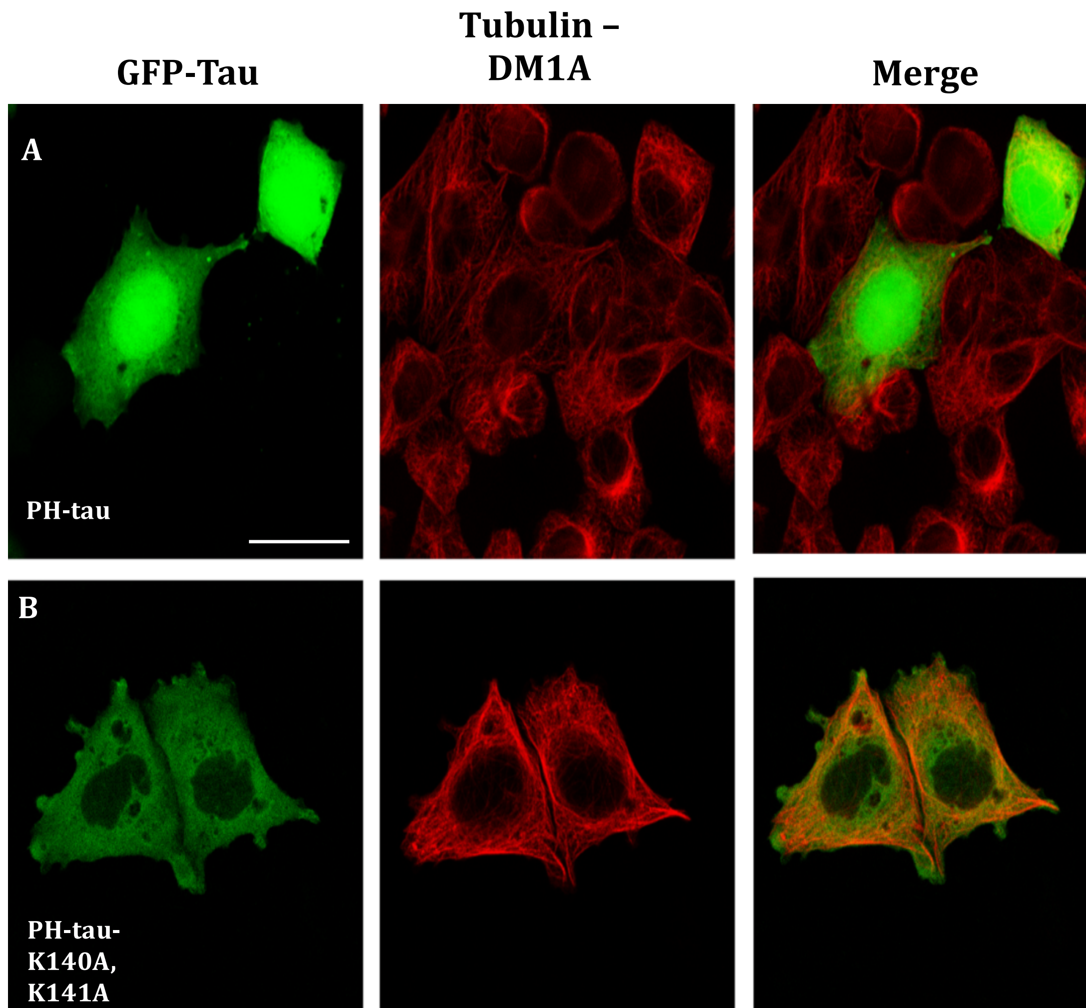


Figure 4.2 – Elimination of the putative importin binding site keeps tau out of the nucleus - Cells were transfected with tau containing either the intact (PH-tau) or the eliminated importin binding site (PH-tau-K140A/K141A). GFP-fused tau vectors were generated and are shown in this figure. The cells were fixed 48 hours post transfection and immunostained against tubulin. When the importin binding site is eliminated (B), tau is no longer able to enter the nucleus of the cells. When the site is intact, tau is found in the cell nucleus (A). Additionally, the cells that express the intact importin binding site begin to round up and loose their cytoplasmic space (A). It is important to note that in both B, even when tau is prevented from entering the nucleus, it still does not associate will with the microtubule structure of the cells. It can be seen free-floating in the cytoplasmic space. Scale bar for all frames is 20μm.

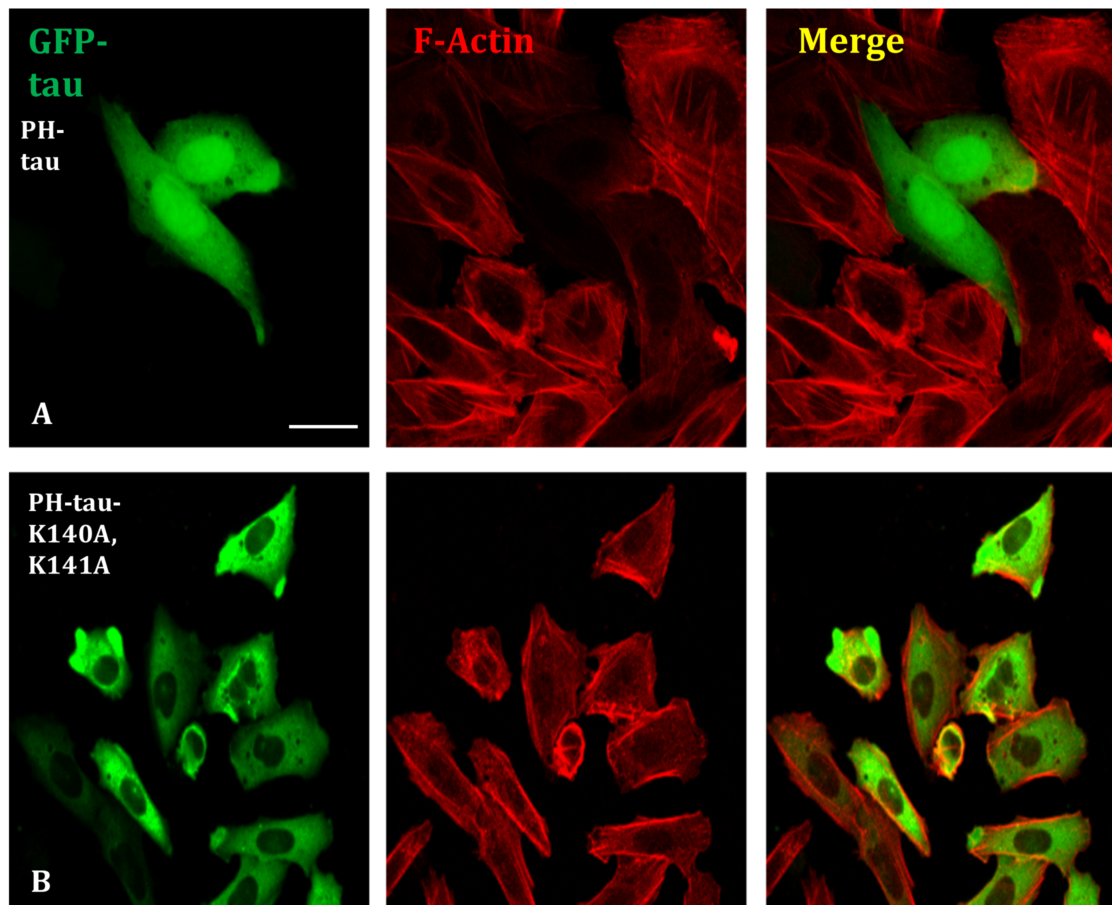


Figure 4.3 – Preventing tau from entering the nucleus does not inhibit PH-tau effect on the F-actin - We have shown that the presence of PH-tau has caused a breakdown in the cellular F-actin. In order to see if the presence of tau in the nucleus played a role in the F-actin breakdown, we transfected CHO cells with both PH-tau and PH-tau-K140A/K141A constructs and later stained for F-actin using rhodamine-phalloidin. A and B have actin that is less fibrillar and punctate in nature. This suggests that inhibiting tau from entering the nucleus does not improve the overall heath of the actin cytoskeleton. Scale bar for all frames is 20μm.

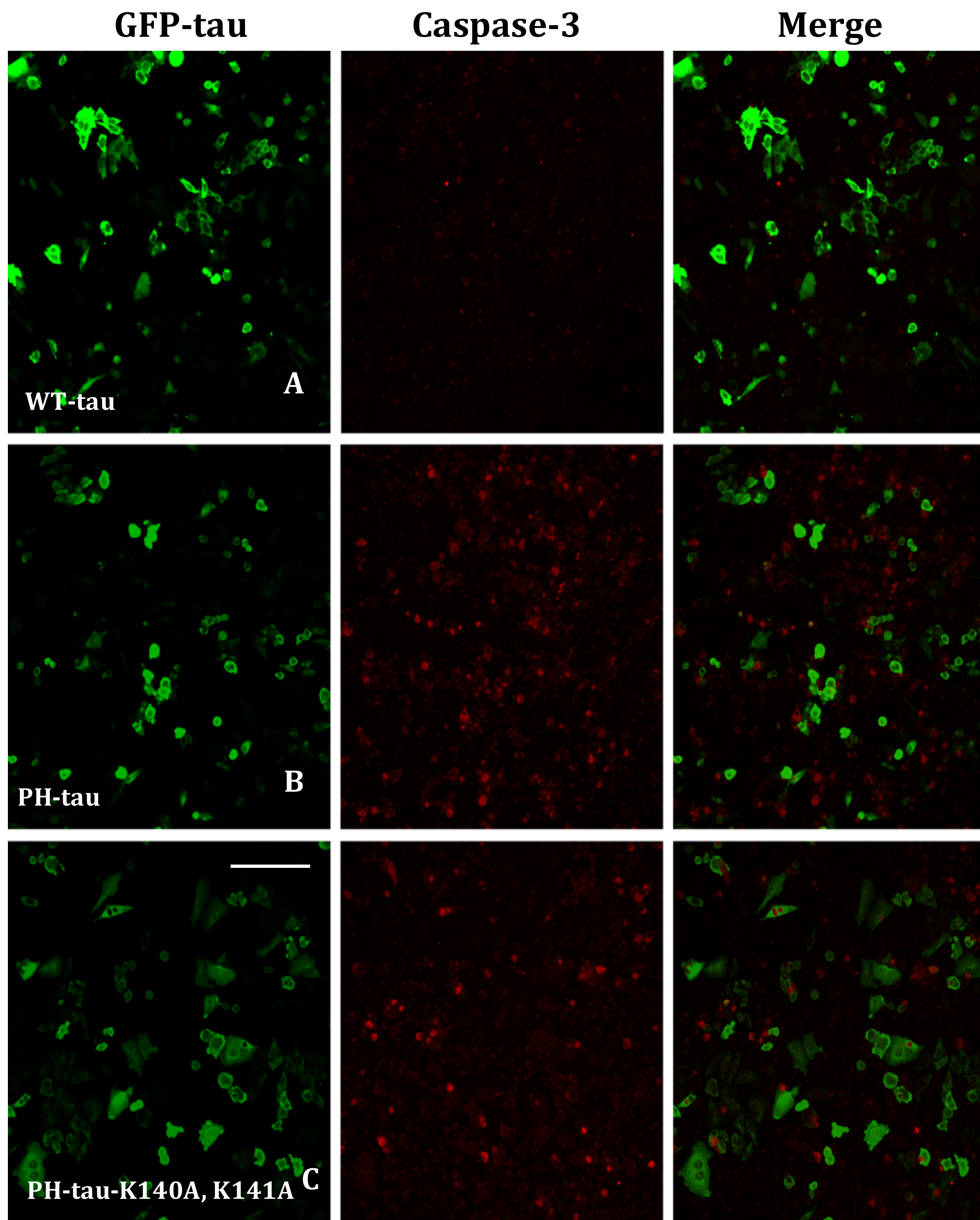


Figure 4.4 – Preventing tau from entering the nucleus does not inhibit PH-tau effect on culture wide caspase 3 activation – It has been previosuly shown that there is an increase in caspase 3 activation in cells that are expressing PH-tau. In addition, we have found that some cells not transfected with the tau protein are also positive for caspase activation suggesting that tau positive cells may be triggering apoptosis induction in neighboring cells. To see if nuclear translocation plays a role in caspase 3 activation, CHO cells were transfected with WT-tau (A) PH-tau (B) and PH-tau-K140A/K141A. GFP-tau, fixed and imunostained for active caspase 3. It is evident that the elimination of the importin binding site does not change PH-tau's ability to induce caspase 3 activation. Scale bar is 50μm

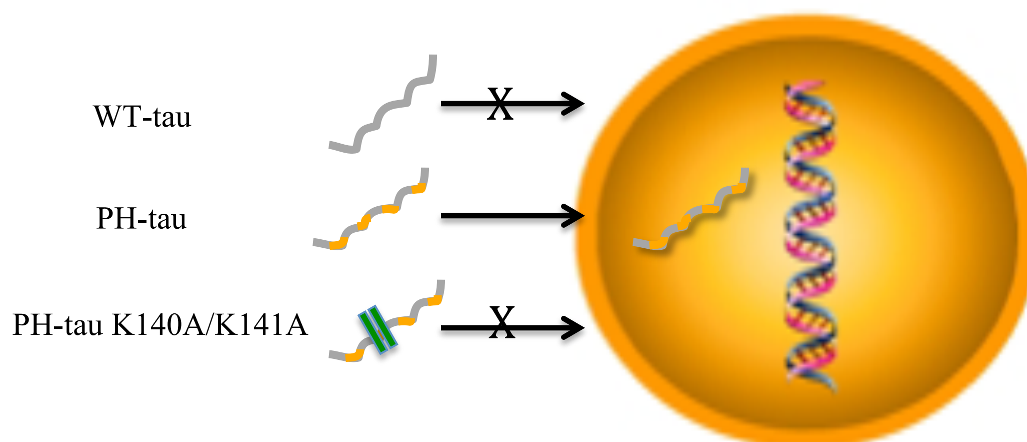


Figure 4.5 – PH-tau can no longer enter the nucleus when the importin binding site is eliminated – In this summary diagram, it is shown that PH-tau is no longer able to enter the nucleus when the importin binding site is mutated.

CHAPTER FIVE

Final Conclusions and Future Directions

5.1 - Neurodegenerative diseases are at an all time high and sill increasing

According to the World Health Organization, Alzheimer's disease is increasing due to an ever-increasing life span. This disease will overtake cancer and be the second leading cause of death (Nowrangi et al., 2011). Additionally, chronic traumatic encephalopathy can be caused by contact sports such as boxing and American football. These patients present with cognitive deficiencies after evens just a short time participating in the sport. These problems develop after repeated head trauma often seen in these sports (Baugh et al., 2012; Stern et al., 2011).

The hyperphosphorylated form of tau has been detected in deficient mice after brain injury (Genis et al., 2000). Neurodegenerative diseases are prevalent into todays society and therefore continued research into the field is critical.

5.2 - Cellular pathologies detected and Okadaic acid treatment

This work has demonstrated that when the amino acid residues T212, T231 and S262 of tau protein are hyperphosphorylated, both with or without the FTDP-17 mutation, lead to several cellular pathologies in addition to microtubule disruption. These pathologies include membrane zeiosis, intracellular vacuole formation, protein aggregation, depolymerization of F-actin, and tau translocation into the nucleus.

The use of a tau, pseudophosphorylated at the above mentioned sites, could raise question if the pathologies seen can be caused by actual phosphorylation at these amino acids. To study this, okadaic acid treatment was utilized. This chemical blocks the activity of phosphatases and therefore cause wild type tau to become and stay hyperphosphorylated. This treatment with okadaic acid demonstrated that actual

hyperphosphorylation of tau leads to the same pathological cellular responses, suggesting that pseudophosphorylation, at least at the sites studied here, is a reliable model to begin to study the effect of tau hyperphosphorylation. Furthermore, the use of phosphor-specific antibodies demonstrated that each of the three above mentioned sites were phosphorylated when treated with the okadaic acid.

5.3 - Membrane Blebbing

Membrane blebbing is a normal and healthy occurrence that occurs in many cell types. Usually, these blebs are taken back into the cell. It is evident that when cells are expressing PH-tau, these blebs are released from the cells into the extracellular environment and are found throughout the culture environment. Additionally, the presence of tau negative, but caspase positive cells, suggests that there is some form of disease progression as cell death is being transferred to other, non-transfected cells in the culture. This idea was proposed in our earlier work (Alonso et al., 1996). This is in support of the prion-like nature of tau protein, which has been shown in several other studies.

In recent literature, it has been shown that tau not only interacts with the plasma membrane, but that the hyperphosphorylated form also contributes to the disruption of the cell membrane (Jones et al., 2012; Pooler and Hanger, 2010). Jones has shown that tau interacts with anionic lipids and triggers protein aggregation. Additionally, membrane permeabilization is a proposed participant in the mechanism that makes aggregated tau toxic.

It has been shown through detailed stereological investigations of the entorhinal cortex of Alzheimer's disease patients post-mortem that the cells of this region tend of be smaller in size and irregularly shaped (Artacho-Pérula, 2007). The CHO cells investigated in this work also appear to have the same decrease in size and the irregular shape. The CHO cells expressing the PH-tau were rounded, and often not well spread out and not adhered well to the culture surface.

Additionally, there is ever increasing evidence that tau can function like a prion (Hall and Patuto, 2012). Kfoury and colleagues have shown that tau pathology can be spread when surrounding cells in culture pick up aggregated tau in cell culture (Kfoury et al., 2012). Furthermore, it has been demonstrated that tau associated with exosomes may play a role in disease transmission (Saman et al., 2012). This type of transmission also seems to be apparent in amyloid beta mediated degeneration (Jucker and Walker, 2011).

Further work will be done to investigate the ability of this specific form of pseudophosphorylated tau to be able to transfer the toxic condition through spread of the pathological protein. Recombinant PH-tau will be purified and placed on healthy cells to see if caspase activation and other apoptotic markers are present. Since PH-tau is known to cause caspase activation in non-expressing cells in a transient transfection system, this experiment will determine if free floating PH-tau can induce toxicity form the extracellular environment.

If the tau is able to cause a toxic reaction in cultured cells, the purified PH-tau will also be injected into the brains of non-transgenic mice to study the effects the free protein has on the central nervous system. This may prove to be a valuable system in which to study MAP sequestration and the progression of tau induced neurodegeneration.

The membrane blebs will be harvested from the cultures and further studied. These blebs alone will be processed for transmission electron microscopy and tau will be labeled with immunogold. The ultrastructural analysis of tau's distribution in these vesicles will determine is tau is present in the blebs and if tau is associated with the membrane or in the cytosolic interior of the structure. Additionally, these cells will be placed on non-transfected CHO cells to determine if tau is able to induce apoptosis. Immunogold will also be done on cells expressing PH-tau to determine the proteins distribution and detect possible aggregation.

Short term live cell imaging was critical in the identification of the membrane blebbing process. Future work will involve and long term live cell imaging over the course of several days will be used to analyze in real time the degenerative process that occurs. Imaging over a longer period of time, in conjunction with vital fluorescent dyes, will allow for tracking of the breakdown of microtubules, activation of capases in the cells and tracking the movement of several intracellular organelles.

Future work will also involve a neuronal system. Using the lab's recently developed inducible transgenic mouse model, primary cultures will be created and PH-tau expression can be induced. In these neuronal cultures, the presence of membrane blebbing can be analyzed in neurons. Through the use of live imaging, again different organelles can be tacked and the microtubule breakdown process can be further analyzed in a neuronal system.

5.4 - Protein Aggregation

The cells expressing the PH-tau constructs in this study also came up positive for intracellular aggregates by thioflavin S staining. This positive result is further supported

through the detection of fibrils under the transmission electron microscope. This data is very interesting because it begins to shed light on which specific sites of phosphorylation are important to drive tau aggregation. Furthermore, the presence of aggregated protein is co-localized with tau expressing cells when immunocytochemically stained for the presence of tau and post-immuno-stained with thioflavin S.

Thioflavin staining will be used in the inducible transgenic mouse model will be done to see *in vivo* if tau is aggregating in the mouse brain.

5.5 - Depolymerization of F-actin by PH-tau

The actin cytoskeleton plays a major role in the structure of a cell by providing a great deal of support at the inner surface of the plasma membrane. It has been demonstrated that F-actin directly interacts with NESH, a protein that is part of the Abi gene family that regulates cell motility. This protein is expressed predominantly in the brain, and has been shown to play a role in new synapse formation (Bae et al., 2012a). It has been shown that NESH levels in synaptic buotons is regulated by association with F-actin. It has been seen that the disruption of F-actin in leads to a re-distribution of NESH out of the synapses and it is believed that this may play a role in the synaptic plasticity (Bae et al., 2012b). This study has shown that hyperphosphorylated tau appears to cause a depolymerization of the F-actin. It has been shown in former works that hyperphosphorylated tau also causes a retraction of neurites (Dubey et al., 2008). Without the presence of F-actin in these PH-tau cells, the change in cellular distribution on NESH may be a possible mechanism of tau neurodegeneration.

Future work will analyze the expression of NESH in the lab's inducible PH-tau expressing mouse model. The presence of NESH in the synaptic terminals could provide mechanistic insight into synapse degeneration in tau induced neurodegeneration.

Cofilin, a major actin depolymerization molecule found in the central nervous system, has been shown to be responsible for the production of actin cofilin rods in Alzheimer's disease and that these rods also associate themselves with the microtubule binding domain on MAPs (Whiteman et al., 2011). Furthermore, the formation of actin cofilin rods plays a role in dendrite microtubule destabilization and contributes to compromised cellular transport, which contribute to synaptic loss (Cichon et al., 2012). The present study does not look for the presence of actin cofilin rods, just actin stress fibers. It has been shown that phalloidin staining does not label actin cofilin rods (Nishida et al., 1987).

Since it has been shown that these structures can lead to neurodegeneration, future work will look into the presence of actin cofilin rods within PH-tau expressing cells, which appear to be void of polymerized actin, but posses what appears to be a normal healthy amount of G-actin. The design of the present study is limited to analyze this question because CHO cells are not of neuronal origin and therefore will not express cofilin. The transgenic mouse line expressing the same PH-tau used in this study, will be used to investigate the actin cofilin rods both *in vivo* and in primary culture models.

Additional work has shown that actin plays a role in exocytosis regulation. In a recent study by Wang and Richards, they have shown that a disruption of the actin cytoskeleton causes a decrease in vesicle movement (Wang et al., 2010b). In our previous work on membrane blebbing, tied with actin stress fiber loss, there may be a correlation

between the lose of actin and the uncontrolled membrane blebbing is actin does in fact play a role in exocytosis regulation. It has recently been demonstrated that actin plays a major role in the coating of exocytotic vesicles and regulates the process as a whole (Nightingale et al., 2012). Without the presence of dynamic actin, the release of exocytotic blebs could occur uncontrollably, which is what we have seen in our earlier work examining excessive membrane zeiosis. The zeiotic processes may be a result of the loss of dynamic F-actin in the presence of PH-tau.

5.6 - Nuclear localization signal on tau binds to importin and is responsible for PH-tau's presence in the nucleus

There is a nuclear localization signal on tau that is able to bind to importin when this protein in hyperphosphorylated. The elimination of this site, by site directed mutagenesis, does prevent tau from entering the nucleus of the cells. Additionally, keeping PH-tau out of the nucleus allows the cells to maintain a healthy microtubule network and stay firmly attached to the culture surface.

However, keeping PH-tau out of the nucleus does not stop the activation of caspase 3 in tau expressing and surrounding cells in culture nor does it stop the breakdown of the actin microfilaments.

More investigation will be required to analyze how PH-tau's presence in the nucleus adversely affects the stability of the membrane and the microtubule networks. Additionally, investigation into the specific importins that are involved in this process both in the CHO cell model as well as in primary cultures from our laboratory's inducible PH-tau expressing mouse. Being able to understand which importins may be overexpressed when PH-tau is present and to which importin PH-tau specifically binds

would prove to be valuable information to further investigate PH-tau and its role in the nucleus. Additionally, short term and long term live cell imaging will be prove useful in determining the dynamics of the microtubules and tracking the degenerative process.

5.7 – What doors does this work open for future research in tau?

This work has begun to investigate a potential mechanism of disease transmission. There is mounting evidence that supports tau being a prion-like protein and the membrane blebbing may pose a potential mechanism for disease transmission. Additionally, this work has begun using live cell imaging to track the degenerative process induced by tau hyperphosphorylation. Continued exploration using this technique to track the degenerative process will allow for a better understanding of tau induced neurodegeneration and how its progression may be slowed down. This work demonstrates that certain serine or threonine sites are more critical when hyperphosphorylated. Work to target the kinases that specifically phosphorylate the sites T212, T231 and S262 could lead to a more refined treatment possibility. The model that has been developed here will be very beneficial is analyzing new treatments.

Furthermore, the breakdown of the actin cytoskeletal components could play a role synapse degradation by way of NESH and cofilin interactions with tau. This work has shown that hyperphosphorylated tau does cause a depolymerization of the F-actin. Further work into the mechanism by which tau causes this breakdown will be critical. Additionally, the role that specific phosphorylation sites for FTDP-17 mutations play will also be critical. Although stopping tau from entering the nucleus does not change the

disease, further investigation into the role that this protein plays inside the nucleus will be critical.

References

Alenghat, T., K. Meyers, S.E. Mullican, K. Leitner, A. Adeniji-Adele, J. Avila, M. Bucan, R.S. Ahima, K.H. Kaestner, and M.A. Lazar. 2008. Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. *Nature*. 456:997-1000.

Alonso, A., T. Zaidi, M. Novak, I. Grundke-Iqbal, and K. Iqbal. 2001a. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. *Proc Natl Acad Sci U S A*. 98:6923-6928.

Alonso, A., T. Zaidi, M. Novak, I. Grundke-Iqbal, and K. Iqbal. 2001b. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 98:6923-6928.

Alonso, A.C., I. Grundke-Iqbal, and K. Iqbal. 1996. Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nature Medicine*. 2:783-787.

Alonso, A.C., B. Li, I. Grundke-Iqbal, and K. Iqbal. 2008. Mechanism of tau-induced neurodegeneration in Alzheimer disease and related tauopathies. *Curr Alzheimer Res*. 5:375-384.

Alonso, A.C., T. Zaidi, I. Grundke-Iqbal, and K. Iqbal. 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 91:5562-5566.

Alonso, A.D., J. Diclerico, B. Li, C.P. Corbo, M.E. Alaniz, I. Grundke-Iqbal, and K. Iqbal. 2010. Phosphorylation of tau at Thr212, Thr231 and Ser262 combined and not individually causes neurodegeneration. *J Biol Chem*. 285:30851-30860.

Alonso, A.D., I. Grundke-Iqbal, H.S. Barra, and K. Iqbal. 1997. Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 94:298-303.

Alonso, A.D., T. Zaidi, M. Novak, H.S. Barra, I. Grundke-Iqbal, and K. Iqbal. 2001c. Interaction of tau isoforms with Alzheimer's disease abnormally hyperphosphorylated tau and in vitro phosphorylation into the disease-like protein. *The Journal Of Biological Chemistry*. 276:37967-37973.

Alonso, A.d.C., B. Li, I. Grundke-Iqbal, and K. Iqbal. 2006. Polymerization of hyperphosphorylated tau into filaments eliminates its inhibitory activity. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 103:8864-8869.

Alonso, A.d.C., A. Mederlyova, M. Novak, I. Grundke-Iqbal, and K. Iqbal. 2004. Promotion of hyperphosphorylation by frontotemporal dementia tau mutations. *The Journal Of Biological Chemistry*. 279:34873-34881.

Amenta, F., L. Parnetti, V. Gallai, and A. Wallin. 2001. Treatment of cognitive dysfunction associated with Alzheimer's disease with cholinergic precursors. Ineffective treatments or inappropriate approaches? *Mechanisms Of Ageing And Development*. 122:2025-2040.

Amorim, M.A., C. Guerra-Araiza, O. Pernia, E.S.E.F. da Cruz, and L.M. Garcia-Segura. 2010. Progesterone regulates the phosphorylation of protein phosphatases in the brain. *J Neurosci Res*. 88:2826-2832.

Andorfer, C., C.M. Acker, Y. Kress, P.R. Hof, K. Duff, and P. Davies. 2005. Cell-cycle reentry and cell death in transgenic mice expressing nonmutant human tau isoforms. *J Neurosci*. 25:5446-5454.

Arnold, C.S., G.V. Johnson, R.N. Cole, D.L. Dong, M. Lee, and G.W. Hart. 1996. The microtubule-associated protein tau is extensively modified with O-linked N-acetylglucosamine. *The Journal Of Biological Chemistry*. 271:28741-28744.

Arrasate, M., S. Mitra, E.S. Schweitzer, M.R. Segal, and S. Finkbeiner. 2004. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature*. 431:805-810.

Aylett, C.H., J. Lowe, and L.A. Amos. 2011. New insights into the mechanisms of cytomotive actin and tubulin filaments. *International review of cell and molecular biology*. 292:1-71.

Azorsa, D.O., R.H. Robeson, D. Frost, B. Meec hoovet, G.R. Brautigam, C. Dickey, C. Beaudry, G.D. Basu, D.R. Holz, J.A. Hernandez, K.M. Bisanz, L. Gwinn, A. Grover, J. Rogers, E.M. Reiman, M. Hutton, D.A. Stephan, S. Mousses, and T. Dunckley. 2010. High-content siRNA screening of the kinome identifies kinases involved in Alzheimer's disease-related tau hyperphosphorylation. *BMC Genomics*. 11:25.

Bacskai, B.J., S.T. Kajdasz, R.H. Christie, C. Carter, D. Games, P. Seubert, D. Schenk, and B.T. Hyman. 2001. Imaging of amyloid-beta deposits in brains of living mice permits direct observation of clearance of plaques with immunotherapy. *Nature Medicine*. 7:369-372.

Bae, J., B.H. Sung, I.H. Cho, S.M. Kim, and W.K. Song. 2012a. NESH regulates dendritic spine morphology and synapse formation. *PloS one*. 7:e34677.

Bae, J., B.H. Sung, I.H. Cho, and W.K. Song. 2012b. F-actin-dependent regulation of NESH dynamics in rat hippocampal neurons. *PloS one*. 7:e34514.

Bancher, C., C. Brunner, H. Lassmann, H. Budka, K. Jellinger, G. Wiche, F. Seitelberger, I. Grundke-Iqbal, K. Iqbal, and H.M. Wisniewski. 1989. Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. *Brain Research*. 477:90-99.

Bancher, C., I. Grundke-Iqbal, K. Iqbal, V.A. Fried, H.T. Smith, and H.M. Wisniewski. 1991. Abnormal phosphorylation of tau precedes ubiquitination in neurofibrillary pathology of Alzheimer disease. *Brain Research*. 539:11-18.

Bard, F., C. Cannon, R. Barbour, R.L. Burke, D. Games, H. Grajeda, T. Guido, K. Hu, J. Huang, K. Johnson-Wood, K. Khan, D. Kholodenko, M. Lee, I. Lieberburg, R. Motter, M. Nguyen, F. Soriano, N. Vasquez, K. Weiss, B. Welch, P. Seubert, D. Schenk, and T. Yednock. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nature Medicine*. 6:916-919.

Baugh, C.M., J.M. Stamm, D.O. Riley, B.E. Gavett, M.E. Shenton, A. Lin, C.J. Nowinski, R.C. Cantu, A.C. McKee, and R.A. Stern. 2012. Chronic traumatic encephalopathy: neurodegeneration following repetitive concussive and subconcussive brain trauma. *Brain imaging and behavior*. 6:244-254.

Ben-Yaakov, K., and M. Fainzilber. 2009. Retrograde injury signaling in lesioned axons. *Results and problems in cell differentiation*. 48:327-338.

Bennecib, M., C.X. Gong, I. Grundke-Iqbal, and K. Iqbal. 2001. Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356. *FEBS Letters*. 490:15-22.

Bertrand, J., P. Senechal, M. Zummo-Soucy, V. Plouffe, and N. Leclerc. 2010. The formation of tau pathological phospho-epitopes in the axon is prevented by the dephosphorylation of selective sites in primary hippocampal neurons over-expressing human tau. *J Neurochem*. 114:1353-1367.

Bhattacharya, R., and F. Cabral. 2004. A ubiquitous beta-tubulin disrupts microtubule assembly and inhibits cell proliferation. *Molecular Biology Of The Cell*. 15:3123-3131.

Blacker, D., and R.E. Tanzi. 1998. The genetics of Alzheimer disease: current status and future prospects. *Archives Of Neurology*. 55:294-296.

Blard, O., S. Feuillette, J. Bou, B. Chaumette, T. Frébourg, D. Campion, and M. Lecourtois. 2007. Cytoskeleton proteins are modulators of mutant tau-induced neurodegeneration in Drosophila. *Human Molecular Genetics*. 16:555-566.

Bleckert, A., H. Photowala, and S. Alford. 2012. Dual pools of actin at presynaptic terminals. *Journal of neurophysiology*. 107:3479-3492.

Boimel, M., N. Grigoriadis, A. Lourbopoulos, E. Haber, O. Abramsky, and H. Rosenmann. 2010. Efficacy and safety of immunization with phosphorylated tau against neurofibrillary tangles in mice. *Experimental Neurology*. 224:472-485.

Bolós, M., S. Fernandez, and I. Torres-Aleman. 2010. Oral administration of a GSK3 inhibitor increases brain insulin-like growth factor I levels. *The Journal Of Biological Chemistry*. 285:17693-17700.

Bondareff, W., C.Q. Mountjoy, M. Roth, and D.L. Hauser. 1989. Neurofibrillary degeneration and neuronal loss in Alzheimer's disease. *Neurobiology Of Aging*. 10:709-715.

Brandt, R., A. Gergou, I. Wacker, T. Fath, and H. Hutter. 2009. A Caenorhabditis elegans model of tau hyperphosphorylation: induction of developmental defects by transgenic overexpression of Alzheimer's disease-like modified tau. *Neurobiology Of Aging*. 30:22-33.

Brandt, R., G. Lee, D.B. Teplow, D. Shalloway, and M. Abdel-Ghany. 1994. Differential effect of phosphorylation and substrate modulation on tau's ability to promote microtubule growth and nucleation. *The Journal Of Biological Chemistry*. 269:11776-11782.

Brandt, R., J. Léger, and G. Lee. 1995. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *The Journal Of Cell Biology*. 131:1327-1340.

Cai, H., Y. Wang, D. McCarthy, H. Wen, D.R. Borchelt, D.L. Price, and P.C. Wong. 2001. BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nature Neuroscience*. 4:233-234.

Cash, A.D., G. Aliev, S.L. Siedlak, A. Nunomura, H. Fujioka, X. Zhu, A.K. Raina, H.V. Vinters, M. Tabaton, A.B. Johnson, M. Paula-Barbosa, J. Avíla, P.K. Jones, R.J. Castellani, M.A. Smith, and G. Perry. 2003. Microtubule reduction in Alzheimer's

disease and aging is independent of tau filament formation. *The American Journal Of Pathology*. 162:1623-1627.

Chang, E., S. Kim, H. Yin, H.N. Nagaraja, and J. Kuret. 2008. Pathogenic missense MAPT mutations differentially modulate tau aggregation propensity at nucleation and extension steps. *J Neurochem*. 107:1113-1123.

Cho, C., and R.D. Vale. 2012. The mechanism of dynein motility: insight from crystal structures of the motor domain. *Biochim Biophys Acta*. 1823:182-191.

Chu, C.T., E.D. Plowey, Y. Wang, V. Patel, and K.L. Jordan-Sciutto. 2007. Location, location, location: altered transcription factor trafficking in neurodegeneration. *Journal of neuropathology and experimental neurology*. 66:873-883.

Cichon, J., C. Sun, B. Chen, M. Jiang, X.A. Chen, Y. Sun, Y. Wang, and G. Chen. 2012. Cofilin aggregation blocks intracellular trafficking and induces synaptic loss in hippocampal neurons. *J Biol Chem*. 287:3919-3929.

Cowan, C.M., T. Bossing, A. Page, D. Shepherd, and A. Mudher. 2010. Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau in vivo. *Acta Neuropathol*. 120:593-604.

Cras, P., M.A. Smith, P.L. Richey, S.L. Siedlak, P. Mulvihill, and G. Perry. 1995. Extracellular neurofibrillary tangles reflect neuronal loss and provide further evidence of extensive protein cross-linking in Alzheimer disease. *Acta Neuropathologica*. 89:291-295.

Crowther, R.A., and M. Goedert. 2000. Abnormal tau-containing filaments in neurodegenerative diseases. *Journal Of Structural Biology*. 130:271-279.

Crowther, R.A., O.F. Olesen, R. Jakes, and M. Goedert. 1992. The microtubule binding repeats of tau protein assemble into filaments like those found in Alzheimer's disease. *FEBS Letters*. 309:199-202.

Cummings, J.L., H.V. Vinters, G.M. Cole, and Z.S. Khachaturian. 1998. Alzheimer's disease: etiologies, pathophysiology, cognitive reserve, and treatment opportunities. *Neurology*. 51:S2-17; discussion S65-17.

Das, P., V. Howard, N. Loosbrock, D. Dickson, M.P. Murphy, and T.E. Golde. 2003. Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma-/- knock-out mice. *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience*. 23:8532-8538.

De Kimpe, L., and W. Scheper. 2010. From Alpha to Omega with Abeta: Targeting the Multiple Molecular Appearances of the Pathogenic Peptide in Alzheimer's Disease. *Curr Med Chem*. 17:198-212.

de Silva, R., and M. Farrer. 2002. Tau neurotoxicity without the lesions: a fly challenges a tangled web. *Trends in neurosciences*. 25:327-329.

De Strooper, B., R. Vassar, and T. Golde. 2010. The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat Rev Neurol*. 6:99-107.

Deture, M., C. Hicks, and L. Petrucelli. 2010. Targeting Heat Shock Proteins in Tauopathies. *Current Alzheimer Research*.

DeTure, M., L.-W. Ko, C. Easson, and S.-H. Yen. 2002. Tau assembly in inducible transfectants expressing wild-type or FTDP-17 tau. *The American Journal Of Pathology*. 161:1711-1722.

Dias-Santagata, D., T.A. Fulga, A. Duttaroy, and M.B. Feany. 2007. Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *The Journal Of Clinical Investigation*. 117:236-245.

Ding, H., P.J. Dolan, and G.V. Johnson. 2008. Histone deacetylase 6 interacts with the microtubule-associated protein tau. *J Neurochem*. 106:2119-2130.

DiTella, M., F. Feiguin, G. Morfini, and A. Cáceres. 1994. Microfilament-associated growth cone component depends upon Tau for its intracellular localization. *Cell Motility And The Cytoskeleton*. 29:117-130.

Dixit, R., J.L. Ross, Y.E. Goldman, and E.L.F. Holzbaur. 2008. Differential regulation of dynein and kinesin motor proteins by tau. *Science (New York, N.Y.)*. 319:1086-1089.

Dodel, R., F. Neff, C. Noelker, R. Pul, Y. Du, M. Bacher, and W. Oertel. 2010. Intravenous immunoglobulins as a treatment for Alzheimer's disease: rationale and current evidence. *Drugs*. 70:513-528.

Drewes, G., B. Trinczek, S. Illenberger, J. Biernat, G. Schmitt-Ulms, H.E. Meyer, E.M. Mandelkow, and E. Mandelkow. 1995. Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. *The Journal Of Biological Chemistry*. 270:7679-7688.

Dubey, M., P. Chaudhury, H. Kabiru, and T.B. Shea. 2008. Tau inhibits anterograde axonal transport and perturbs stability in growing axonal neurites in part by displacing kinesin cargo: neurofilaments attenuate tau-mediated neurite instability. *Cell Motil Cytoskeleton*. 65:89-99.

Dudek, S.M., and G.V. Johnson. 1993. Transglutaminase catalyzes the formation of sodium dodecyl sulfate-insoluble, Alz-50-reactive polymers of tau. *Journal Of Neurochemistry*. 61:1159-1162.

Ebadi, M., S.K. Sharma, P. Ghafourifar, H. Brown-Borg, and H. El Refaey. 2005. Peroxynitrite in the pathogenesis of Parkinson's disease and the neuroprotective role of metallothioneins. *Methods in enzymology*. 396:276-298.

Ebneth, A., R. Godemann, K. Stamer, S. Illenberger, B. Trinczek, and E. Mandelkow. 1998. Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *The Journal Of Cell Biology*. 143:777-794.

Engel, T., P. Goni-Oliver, J.J. Lucas, J. Avila, and F. Hernandez. 2006. Chronic lithium administration to FTDP-17 tau and GSK-3beta overexpressing mice prevents tau hyperphosphorylation and neurofibrillary tangle formation, but pre-formed neurofibrillary tangles do not revert. *J Neurochem*. 99:1445-1455.

Ewald, C.Y., and C. Li. 2010. Understanding the molecular basis of Alzheimer's disease using a Caenorhabditis elegans model system. *Brain Structure & Function*. 214:263-283.

Fasulo, L., G. Ugolini, M. Visintin, A. Bradbury, C. Brancolini, V. Verzillo, M. Novak, and A. Cattaneo. 2000. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. *Journal Of Neurochemistry*. 75:624-633.

Feinstein, S.C., and L. Wilson. 2005. Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediate neuronal cell death. *Biochimica Et Biophysica Acta*. 1739:268-279.

Feuillette, S., L. Miguel, T. Frébourg, D. Campion, and M. Lecourtois. 2010. Drosophila models of human tauopathies indicate that Tau protein toxicity in vivo is mediated by soluble cytosolic phosphorylated forms of the protein. *Journal Of Neurochemistry*. 113:895-903 %U

Frisoni, G.B., M. Pievani, C. Testa, F. Sabattoli, L. Bresciani, M. Bonetti, A. Beltramello, K.M. Hayashi, A.W. Toga, and P.M. Thompson. 2007. The topography of grey matter involvement in early and late onset Alzheimer's disease. *Brain*. 130:720-730.

Fulga, T.A., I. Elson-Schwab, V. Khurana, M.L. Steinhilb, T.L. Spires, B.T. Hyman, and M.B. Feany. 2007. Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo. *Nature Cell Biology*. 9:139-148.

Gamblin, T.C., R.W. Berry, and L.I. Binder. 2003a. Tau polymerization: role of the amino terminus. *Biochemistry*. 42:2252-2257.

Gamblin, T.C., F. Chen, A. Zambrano, A. Abraha, S. Lagalwar, A.L. Guillozet, M. Lu, Y. Fu, F. Garcia-Sierra, N. LaPointe, R. Miller, R.W. Berry, L.I. Binder, and V.L. Cryns. 2003b. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 100:10032-10037.

Gamblin, T.C., M.E. King, J. Kuret, R.W. Berry, and L.I. Binder. 2000. Oxidative regulation of fatty acid-induced tau polymerization. *Biochemistry*. 39:14203-14210.

Genis, L., Y. Chen, E. Shohami, and D.M. Michaelson. 2000. Tau hyperphosphorylation in apolipoprotein E-deficient and control mice after closed head injury. *J Neurosci Res*. 60:559-564.

Goate, A., M.C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, and L. James. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*. 349:704-706.

Goedert, M., R. Jakes, M.G. Spillantini, M. Hasegawa, M.J. Smith, and R.A. Crowther. 1996. Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature*. 383:550-553.

Goedert, M., M.G. Spillantini, N.J. Cairns, and R.A. Crowther. 1992. Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms. *Neuron*. 8:159-168.

Goedert, M., M.G. Spillantini, R.A. Crowther, S.G. Chen, P. Parchi, M. Tabaton, D.J. Lanska, W.R. Markesbery, K.C. Wilhelmsen, D.W. Dickson, R.B. Petersen, and P. Gambetti. 1999. Tau gene mutation in familial progressive subcortical gliosis. *Nature Medicine*. 5:454-457.

Goedert, M., M.G. Spillantini, R. Jakes, D. Rutherford, and R.A. Crowther. 1989. Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron*. 3:519-526.

Gómez de Barreda, E., M. Pérez, P. Gómez Ramos, J. de Cristobal, P. Martín-Maestro, A. Morán, H.N. Dawson, M.P. Vitek, J.J. Lucas, F. Hernández, and J. Avila.

2010. Tau-knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits. *Neurobiology Of Disease*. 37:622-629.
Gomez-Ramos, A., M.A. Smith, G. Perry, and J. Avila. 2004. Tau phosphorylation and assembly. *Acta neurobiologiae experimentalis*. 64:33-39.
Gong, C.X., I. Grundke-Iqbal, Z. Damuni, and K. Iqbal. 1994. Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and -2C and its implication in Alzheimer disease. *FEBS Letters*. 341:94-98.
Gong, C.X., T. Lidsky, J. Wegiel, L. Zuck, I. Grundke-Iqbal, and K. Iqbal. 2000. Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. *The Journal Of Biological Chemistry*. 275:5535-5544.
Gong, C.X., F. Liu, I. Grundke-Iqbal, and K. Iqbal. 2005. Post-translational modifications of tau protein in Alzheimer's disease. *Journal of neural transmission (Vienna, Austria : 1996)*. 112:813-838.
Gong, C.X., T.J. Singh, I. Grundke-Iqbal, and K. Iqbal. 1993. Phosphoprotein phosphatase activities in Alzheimer disease brain. *Journal Of Neurochemistry*. 61:921-927.
Götz, J., F. Chen, J. van Dorpe, and R.M. Nitsch. 2001. Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. *Science (New York, N.Y.)*. 293:1491-1495.
Gotz, J., and N.N. Gotz. 2009. Animal models for Alzheimer's disease and frontotemporal dementia: a perspective. *ASN Neuro*. 1:251-264.
Gozes, I., and I. Divinski. 2007. NAP, a neuroprotective drug candidate in clinical trials, stimulates microtubule assembly in the living cell. *Current Alzheimer Research*. 4:507-509.
Gozes, I., A. Stewart, B. Morimoto, A. Fox, K. Sutherland, and D. Schmeche. 2009. Addressing Alzheimer's disease tangles: from NAP to AL-108. *Current Alzheimer Research*. 6:455-460.
Grundke-Iqbal, I., K. Iqbal, M. Quinlan, Y.C. Tung, M.S. Zaidi, and H.M. Wisniewski. 1986a. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *The Journal Of Biological Chemistry*. 261:6084-6089.
Grundke-Iqbal, I., K. Iqbal, Y.C. Tung, M. Quinlan, H.M. Wisniewski, and L.I. Binder. 1986b. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 83:4913-4917.
Grundke-Iqbal, I., K. Iqbal, Y.C. Tung, M. Quinlan, H.M. Wisniewski, and L.I. Binder. 1986c. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A*. 83:4913-4917.
Haass, C., and D.J. Selkoe. 1993. Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell*. 75:1039-1042 %U
Hall, G.F., and B.A. Patuto. 2012. Is tau ready for admission to the prion club? *Prion*. 6.
Hanger, D.P., J.C. Betts, T.L. Loviny, W.P. Blackstock, and B.H. Anderton. 1998. New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *Journal Of Neurochemistry*. 71:2465-2476.

Hanger, D.P., H.L. Byers, S. Wray, K.-Y. Leung, M.J. Saxton, A. Seereeram, C.H. Reynolds, M.A. Ward, and B.H. Anderton. 2007. Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *The Journal Of Biological Chemistry*. 282:23645-23654.

Hanz, S., E. Perlson, D. Willis, J.Q. Zheng, R. Massarwa, J.J. Huerta, M. Koltzenburg, M. Kohler, J. van-Minnen, J.L. Twiss, and M. Fainzilber. 2003. Axoplasmic importins enable retrograde injury signaling in lesioned nerve. *Neuron*. 40:1095-1104.

Harada, A., K. Oguchi, S. Okabe, J. Kuno, S. Terada, T. Ohshima, R. Sato-Yoshitake, Y. Takei, T. Noda, and N. Hirokawa. 1994. Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature*. 369:488-491.

Hartmann, D., B. de Strooper, L. Serneels, K. Craessaerts, A. Herreman, W. Annaert, L. Umans, T. Lübke, A. Lena Illert, K. von Figura, and P. Saftig. 2002. The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Human Molecular Genetics*. 11:2615-2624 %U

Hasegawa, M., M.J. Smith, and M. Goedert. 1998. Tau proteins with FTDP-17 mutations have a reduced ability to promote microtubule assembly. *FEBS Letters*. 437:207-210.

He, H.J., X.S. Wang, R. Pan, D.L. Wang, M.N. Liu, and R.Q. He. 2009. The proline-rich domain of tau plays a role in interactions with actin. *BMC cell biology*. 10:81.

Hernandez, F., E. Gomez de Barreda, A. Fuster-Matanzo, J.J. Lucas, and J. Avila. 2010. GSK3: a possible link between beta amyloid peptide and tau protein. *Exp Neurol*. 223:322-325.

Hernández, F., J.D. Nido, J. Avila, and N. Villanueva. 2009. GSK3 inhibitors and disease. *Mini Reviews In Medicinal Chemistry*. 9:1024-1029.

Himmler, A., D. Drechsel, M.W. Kirschner, and D.W. Martin. 1989. Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Molecular And Cellular Biology*. 9:1381-1388.

Hirokawa, N. 2011. From electron microscopy to molecular cell biology, molecular genetics and structural biology: intracellular transport and kinesin superfamily proteins, KIFs: genes, structure, dynamics and functions. *Journal of electron microscopy*. 60 Suppl 1:S63-92.

Ho, L., Z. Xiang, P. Mukherjee, W. Zhang, N. De Jesus, M. Mirjany, S. Yemul, and G.M. Pasinetti. 2001. Gene expression profiling of the tau mutant (P301L) transgenic mouse brain. *Neuroscience Letters*. 310:1-4.

Hong, M., V. Zhukareva, V. Vogelsberg-Ragaglia, Z. Wszolek, L. Reed, B.I. Miller, D.H. Geschwind, T.D. Bird, D. McKeel, A. Goate, J.C. Morris, K.C. Wilhelmsen, G.D. Schellenberg, J.Q. Trojanowski, and V.M. Lee. 1998. Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. *Science (New York, N.Y.)*. 282:1914-1917.

Horiguchi, T., K. Uryu, B.I. Giasson, H. Ischiropoulos, R. LightFoot, C. Bellmann, C. Richter-Landsberg, V.M.Y. Lee, and J.Q. Trojanowski. 2003. Nitration of tau protein is linked to neurodegeneration in tauopathies. *The American Journal Of Pathology*. 163:1021-1031.

Hua, Q., R.Q. He, N. Haque, M.H. Qu, A. del Carmen Alonso, I. Grundke-Iqbal, and K. Iqbal. 2003. Microtubule associated protein tau binds to double-stranded but not single-stranded DNA. *Cellular And Molecular Life Sciences: CMLS*. 60:413-421.

Hutton, M., C.L. Lendon, P. Rizzu, M. Baker, S. Froelich, H. Houlden, S. Pickering-Brown, S. Chakraverty, A. Isaacs, A. Grover, J. Hackett, J. Adamson, S. Lincoln, D. Dickson, P. Davies, R.C. Petersen, M. Stevens, E. de Graaff, E. Wauters, J. van Baren, M. Hillebrand, M. Joosse, J.M. Kwon, P. Nowotny, L.K. Che, J. Norton, J.C. Morris, L.A. Reed, J. Trojanowski, H. Basun, L. Lannfelt, M. Neystat, S. Fahn, F. Dark, T. Tannenberg, P.R. Dodd, N. Hayward, J.B. Kwok, P.R. Schofield, A. Andreadis, J. Snowden, D. Craufurd, D. Neary, F. Owen, B.A. Oostra, J. Hardy, A. Goate, J. van Swieten, D. Mann, T. Lynch, and P. Heutink. 1998. Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*. 393:702-705.

Iijima-Ando, K., L. Zhao, A. Gatt, C. Shenton, and K. Iijima. 2010. A DNA damage-activated checkpoint kinase phosphorylates tau and enhances tau-induced neurodegeneration. *Human Molecular Genetics*. 19:1930-1938.

Ikegami, S., A. Harada, and N. Hirokawa. 2000. Muscle weakness, hyperactivity, and impairment in fear conditioning in tau-deficient mice. *Neuroscience Letters*. 279:129-132.

Iqbal, K., A.C. Alonso, C.X. Gong, S. Khatoon, T.J. Singh, and I. Grundke-Iqbal. 1994. Mechanism of neurofibrillary degeneration in Alzheimer's disease. *Mol Neurobiol*. 9:119-123.

Iqbal, K., A.d.C. Alonso, S. Chen, M.O. Chohan, E. El-Akkad, C.-X. Gong, S. Khatoon, B. Li, F. Liu, A. Rahman, H. Tanimukai, and I. Grundke-Iqbal. 2005. Tau pathology in Alzheimer disease and other tauopathies. *Biochimica Et Biophysica Acta*. 1739:198-210.

Iqbal, K., and I. Grundke-Iqbal. 1991. Ubiquitination and abnormal phosphorylation of paired helical filaments in Alzheimer's disease. *Molecular Neurobiology*. 5:399-410.

Iqbal, K., I. Grundke-Iqbal, T. Zaidi, P.A. Merz, G.Y. Wen, S.S. Shaikh, H.M. Wisniewski, I. Alafuzoff, and B. Winblad. 1986. Defective brain microtubule assembly in Alzheimer's disease. *Lancet*. 2:421-426.

Iqbal, K., F. Liu, C.X. Gong, C. Alonso Adel, and I. Grundke-Iqbal. 2009. Mechanisms of tau-induced neurodegeneration. *Acta Neuropathol*. 118:53-69.

Irwin, D.J., T.J. Cohen, M. Grossman, S.E. Arnold, S.X. Xie, V.M. Lee, and J.Q. Trojanowski. 2012. Acetylated tau, a novel pathological signature in Alzheimer's disease and other tauopathies. *Brain*. 135:807-818.

Jenkins, S.M., and G.V. Johnson. 1997. Phosphorylation of microtubule-associated protein tau on Ser 262 by an embryonic 100 kDa protein kinase. *Brain Research*. 767:305-313.

Jicha, G.A., E. Lane, I. Vincent, L. Otvos, R. Hoffmann, and P. Davies. 1997. A conformation- and phosphorylation-dependent antibody recognizing the paired helical filaments of Alzheimer's disease. *Journal Of Neurochemistry*. 69:2087-2095.

Jones, E.M., M. Dubey, P.J. Camp, B.C. Vernon, J. Biernat, E. Mandelkow, J. Majewski, and E.Y. Chi. 2012. Interaction of tau protein with model lipid membranes

induces tau structural compaction and membrane disruption. *Biochemistry*. 51:2539-2550.

Jope, R.S., C.J. Yuskaitis, and E. Beurel. 2007. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochemical Research*. 32:577-595.

Jucker, M., and L.C. Walker. 2011. Pathogenic protein seeding in Alzheimer disease and other neurodegenerative disorders. *Ann Neurol*. 70:532-540.

Kamemura, K., B.K. Hayes, F.I. Comer, and G.W. Hart. 2002. Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. *The Journal Of Biological Chemistry*. 277:19229-19235.

Kampers, T., P. Friedhoff, J. Biernat, E.M. Mandelkow, and E. Mandelkow. 1996. RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments. *FEBS Letters*. 399:344-349.

Karbowski, M., and A. Neutzner. 2012. Neurodegeneration as a consequence of failed mitochondrial maintenance. *Acta Neuropathol*. 123:157-171.

Kfoury, N., B.B. Holmes, H. Jiang, D.M. Holtzman, and M.I. Diamond. 2012. Trans-cellular Propagation of Tau Aggregation by Fibrillar Species. *J Biol Chem*. 287:19440-19451.

Khatoon, S., I. Grundke-Iqbal, and K. Iqbal. 1992. Brain levels of microtubule-associated protein tau are elevated in Alzheimer's disease: a radioimmuno-slot-blot assay for nanograms of the protein. *Journal Of Neurochemistry*. 59:750-753.

Khatoon, S., I. Grundke-Iqbal, and K. Iqbal. 1995. Guanosine triphosphate binding to beta-subunit of tubulin in Alzheimer's disease brain: role of microtubule-associated protein tau. *Journal Of Neurochemistry*. 64:777-787.

Khlistunova, I., J. Biernat, Y. Wang, M. Pickhardt, M. von Bergen, Z. Gazova, E. Mandelkow, and E.-M. Mandelkow. 2006. Inducible expression of Tau repeat domain in cell models of tauopathy: aggregation is toxic to cells but can be reversed by inhibitor drugs. *J Biol Chem*. 281:1205-1214.

Khurana, V., and M.B. Feany. 2007. Connecting cell-cycle activation to neurodegeneration in Drosophila. *Biochimica Et Biophysica Acta*. 1772:446-456.

Khurana, V., Y. Lu, M.L. Steinhilb, S. Oldham, J.M. Shulman, and M.B. Feany. 2006. TOR-mediated cell-cycle activation causes neurodegeneration in a Drosophila tauopathy model. *Current Biology: CB*. 16:230-241.

Kidd, M. 1963. Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature*. 197:192-193.

Kins, S., P. Kurosinski, R.M. Nitsch, and J. Götz. 2003. Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice. *The American Journal Of Pathology*. 163:833-843.

Kitazawa, M., S. Oddo, T.R. Yamasaki, K.N. Green, and F.M. LaFerla. 2005. Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. *J Neurosci*. 25:8843-8853.

Ko, L.-W., M. DeTure, N. Sahara, R. Chihab, I.E. Vega, and S.-H. Yen. 2005. Recent advances in experimental modeling of the assembly of tau filaments. *Biochimica Et Biophysica Acta*. 1739:125-139.

Köpke, E., Y.C. Tung, S. Shaikh, A.C. Alonso, K. Iqbal, and I. Grundke-Iqbal. 1993. Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. *The Journal Of Biological Chemistry*. 268:24374-24384.

Kovacech, B., and M. Novak. 2010. Tau truncation is a productive posttranslational modification of neurofibrillary degeneration in Alzheimer's disease. *Curr Alzheimer Res*. 7:708-716.

Ksiezak-Reding, H., W.K. Liu, and S.H. Yen. 1992. Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. *Brain Research*. 597:209-219.

Lambourne, S.L., L.A. Sellers, T.G. Bush, S.K. Choudhury, P.C. Emson, Y.-H. Suh, and L.S. Wilkinson. 2005. Increased tau phosphorylation on mitogen-activated protein kinase consensus sites and cognitive decline in transgenic models for Alzheimer's disease and FTDP-17: evidence for distinct molecular processes underlying tau abnormalities. *Molecular And Cellular Biology*. 25:278-293.

Le Corre, S., H.W. Klafki, N. Plesnila, G. Hübinger, A. Obermeier, H. Sahagún, B. Monse, P. Seneci, J. Lewis, J. Eriksen, C. Zehr, M. Yue, E. McGowan, D.W. Dickson, M. Hutton, and H.M. Roder. 2006. An inhibitor of tau hyperphosphorylation prevents severe motor impairments in tau transgenic mice. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 103:9673-9678.

Lee, G., S.T. Newman, D.L. Gard, H. Band, and G. Panchamoorthy. 1998. Tau interacts with src-family non-receptor tyrosine kinases. *Journal Of Cell Science*. 111 (Pt 21):3167-3177.

Lee, G., R. Thangavel, V.M. Sharma, J.M. Litersky, K. Bhaskar, S.M. Fang, L.H. Do, A. Andreadis, G. Van Hoesen, and H. Ksiezak-Reding. 2004. Phosphorylation of tau by fyn: implications for Alzheimer's disease. *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience*. 24:2304-2312.

Lee, H.G., M. Ueda, Y. Miyamoto, Y. Yoneda, G. Perry, M.A. Smith, and X. Zhu. 2006. Aberrant localization of importin alpha1 in hippocampal neurons in Alzheimer disease. *Brain Res*. 1124:1-4.

Levy, S.F., A.C. Leboeuf, M.R. Massie, M.A. Jordan, L. Wilson, and S.C. Feinstein. 2005. Three- and four-repeat tau regulate the dynamic instability of two distinct microtubule subpopulations in qualitatively different manners. Implications for neurodegeneration. *The Journal Of Biological Chemistry*. 280:13520-13528.

Levy-Lahad, E., W. Wasco, P. Poorkaj, D.M. Romano, J. Oshima, W.H. Pettingell, C.E. Yu, P.D. Jondro, S.D. Schmidt, and K. Wang. 1995. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science (New York, N.Y.)*. 269:973-977.

Lewis, J., E. McGowan, J. Rockwood, H. Melrose, P. Nacharaju, M. Van Slegtenhorst, K. Gwinn-Hardy, M. Paul Murphy, M. Baker, X. Yu, K. Duff, J. Hardy, A. Corral, W.L. Lin, S.H. Yen, D.W. Dickson, P. Davies, and M. Hutton. 2000. Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nature Genetics*. 25:402-405.

Li, B., M.O. Chohan, I. Grundke-Iqbal, and K. Iqbal. 2007. Disruption of microtubule network by Alzheimer abnormally hyperphosphorylated tau. *Acta Neuropathologica*. 113:501-511.

Li, L., A. Sengupta, N. Haque, I. Grundke-Iqbal, and K. Iqbal. 2004. Memantine inhibits and reverses the Alzheimer type abnormal hyperphosphorylation of tau and associated neurodegeneration. *FEBS Letters*. 566:261-269.

Li, M., A. Makkinje, and Z. Damuni. 1996a. Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry*. 35:6998-7002.

Li, M., A. Makkinje, and Z. Damuni. 1996b. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *The Journal Of Biological Chemistry*. 271:11059-11062.

Liu, F., I. Grundke-Iqbal, K. Iqbal, and C.-X. Gong. 2005. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *The European Journal Of Neuroscience*. 22:1942-1950.

Liu, F., K. Iqbal, I. Grundke-Iqbal, G.W. Hart, and C.-X. Gong. 2004a. O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 101:10804-10809.

Liu, F., K. Iqbal, I. Grundke-Iqbal, G.W. Hart, and C.X. Gong. 2004b. O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc Natl Acad Sci U S A*. 101:10804-10809.

Liu, F., T. Zaidi, K. Iqbal, I. Grundke-Iqbal, R.K. Merkle, and C.X. Gong. 2002. Role of glycosylation in hyperphosphorylation of tau in Alzheimer's disease. *FEBS Letters*. 512:101-106.

Liu, S.J., J.Y. Zhang, H.L. Li, Z.Y. Fang, Q. Wang, H.M. Deng, C.X. Gong, I. Grundke-Iqbal, K. Iqbal, and J.Z. Wang. 2004c. Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain. *The Journal Of Biological Chemistry*. 279:50078-50088.

Lott, K., and G. Cingolani. 2011. The importin beta binding domain as a master regulator of nucleocytoplasmic transport. *Biochim Biophys Acta*. 1813:1578-1592.

Luo, Y., B. Bolon, S. Kahn, B.D. Bennett, S. Babu-Khan, P. Denis, W. Fan, H. Kha, J. Zhang, Y. Gong, L. Martin, J.C. Louis, Q. Yan, W.G. Richards, M. Citron, and R. Vassar. 2001. Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nature Neuroscience*. 4:231-232.

Mack, T.G., R. Dayanandan, M. Van Slegtenhorst, A. Whone, M. Hutton, S. Lovestone, and B.H. Anderton. 2001. Tau proteins with frontotemporal dementia-17 mutations have both altered expression levels and phosphorylation profiles in differentiated neuroblastoma cells. *Neuroscience*. 108:701-712.

Mah, V.H., T.A. Eskin, A.M. Kazee, L. Lapham, and G.A. Higgins. 1992. In situ hybridization of calcium/calmodulin dependent protein kinase II and tau mRNAs; species differences and relative preservation in Alzheimer's disease. *Brain Research. Molecular Brain Research*. 12:85-94.

Maretzky, T., K. Reiss, A. Ludwig, J. Buchholz, F. Scholz, E. Proksch, B. de Strooper, D. Hartmann, and P. Saftig. 2005. ADAM10 mediates E-cadherin shedding and

regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 102:9182-9187

Matsumura, N., T. Yamazaki, and Y. Ihara. 1999. Stable expression in Chinese hamster ovary cells of mutated tau genes causing frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). *The American Journal Of Pathology*. 154:1649-1656.

Matsushima, K., K. Tokuraku, M.R. Hasan, and S. Kotani. 2012. Microtubule-associated protein 4 binds to actin filaments and modulates their properties. *Journal of biochemistry*. 151:99-108.

Merrick, S.E., J.Q. Trojanowski, and V.M. Lee. 1997. Selective destruction of stable microtubules and axons by inhibitors of protein serine/threonine phosphatases in cultured human neurons. *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience*. 17:5726-5737.

Miller, M.L., and G.V. Johnson. 1995. Transglutaminase cross-linking of the tau protein. *Journal Of Neurochemistry*. 65:1760-1770.

Montejo de Garcini, E., J.C. Díez, and J. Avila. 1986. Quantitation and characterization of tau factor in porcine tissues. *Biochimica Et Biophysica Acta*. 881:456-461.

Moreira, P.I., K. Honda, Q. Liu, M.S. Santos, C.R. Oliveira, G. Aliev, A. Nunomura, X. Zhu, M.A. Smith, and G. Perry. 2005. Oxidative stress: the old enemy in Alzheimer's disease pathophysiology. *Current Alzheimer Research*. 2:403-408.

Morishima, M., and Y. Ihara. 1994. Posttranslational modifications of tau in paired helical filaments. *Dementia (Basel, Switzerland)*. 5:282-288.

Morishima-Kawashima, M., M. Hasegawa, K. Takio, M. Suzuki, K. Titani, and Y. Ihara. 1993. Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. *Neuron*. 10:1151-1160.

Morishima-Kawashima, M., M. Hasegawa, K. Takio, M. Suzuki, H. Yoshida, A. Watanabe, K. Titani, and Y. Ihara. 1995. Hyperphosphorylation of tau in PHF. *Neurobiology Of Aging*. 16:365-371.

Mukrasch, M.D., J. Biernat, M. von Bergen, C. Griesinger, E. Mandelkow, and M. Zweckstetter. 2005. Sites of tau important for aggregation populate {beta}-structure and bind to microtubules and polyanions. *The Journal Of Biological Chemistry*. 280:24978-24986.

Münch, G., W. Deuther-Conrad, and J. Gasic-Milenkovic. 2002. Glycoxidative stress creates a vicious cycle of neurodegeneration in Alzheimer's disease--a target for neuroprotective treatment strategies? *Journal Of Neural Transmission. Supplementum*:303-307 %U

Nayeem, N., F. Kerr, H. Naumann, J. Linehan, S. Lovestone, and S. Brandner. 2007. Hyperphosphorylation of tau and neurofilaments and activation of CDK5 and ERK1/2 in PTEN-deficient cerebella. *Molecular And Cellular Neurosciences*. 34:400-408.

Nightingale, T.D., D.F. Cutler, and L.P. Cramer. 2012. Actin coats and rings promote regulated exocytosis. *Trends in cell biology*. 22:329-337.

Nishida, E., K. Iida, N. Yonezawa, S. Koyasu, I. Yahara, and H. Sakai. 1987. Cofilin is a component of intranuclear and cytoplasmic actin rods induced in cultured cells. *Proc Natl Acad Sci U S A*. 84:5262-5266.

Norlund, M.A., J.M. Lee, G.M. Zainelli, and N.A. Muma. 1999. Elevated transglutaminase-induced bonds in PHF tau in Alzheimer's disease. *Brain Research*. 851:154-163.

Novak, M., R. Jakes, P.C. Edwards, C. Milstein, and C.M. Wischik. 1991. Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 88:5837-5841.

Nowrangi, M.A., V. Rao, and C.G. Lyketsos. 2011. Epidemiology, assessment, and treatment of dementia. *The Psychiatric clinics of North America*. 34:275-294, vii.

Panda, D., J.C. Samuel, M. Massie, S.C. Feinstein, and L. Wilson. 2003. Differential regulation of microtubule dynamics by three- and four-repeat tau: implications for the onset of neurodegenerative disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 100:9548-9553.

Pei, J.-J., C.-X. Gong, W.-L. An, B. Winblad, R.F. Cowburn, I. Grundke-Iqbal, and K. Iqbal. 2003. Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. *The American Journal Of Pathology*. 163:845-858.

Pérez, M., M. Arrasate, E. Montejo De Garcini, V. Muñoz, and J. Avila. 2001. In vitro assembly of tau protein: mapping the regions involved in filament formation. *Biochemistry*. 40:5983-5991.

Perez, M., I. Santa-Maria, E. Gomez de Barreda, X. Zhu, R. Cuadros, J.R. Cabrero, F. Sanchez-Madrid, H.N. Dawson, M.P. Vitek, G. Perry, M.A. Smith, and J. Avila. 2009. Tau--an inhibitor of deacetylase HDAC6 function. *J Neurochem*. 109:1756-1766.

Pérez, M., J.M. Valpuesta, M. Medina, E. Montejo de Garcini, and J. Avila. 1996. Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction. *Journal Of Neurochemistry*. 67:1183-1190.

Pericak-Vance, M.A., J. Grubber, L.R. Bailey, D. Hedges, S. West, L. Santoro, B. Kemmerer, J.L. Hall, A.M. Saunders, A.D. Roses, G.W. Small, W.K. Scott, P.M. Conneally, J.M. Vance, and J.L. Haines. 2000. Identification of Novel Genes in Late-Onset Alzheimer's Disease. *Experimental Gerontology*. 35:1343-1352.

Perry, G., J. Avila, and J. Kinoshita. 2006. Alzheimer's Disease : A Century of Scientific and Clinical Research. IOS Press Amsterdam, , NLD.

Petrucelli, L., D. Dickson, K. Kehoe, J. Taylor, H. Snyder, A. Grover, M. De Lucia, E. McGowan, J. Lewis, G. Prihar, J. Kim, W.H. Dillmann, S.E. Browne, A. Hall, R. Voellmy, Y. Tsuboi, T.M. Dawson, B. Wolozin, J. Hardy, and M. Hutton. 2004. CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. *Human Molecular Genetics*. 13:703-714.

Pooler, A.M., and D.P. Hanger. 2010. Functional implications of the association of tau with the plasma membrane. *Biochemical Society transactions*. 38:1012-1015.

Poorkaj, P., T.D. Bird, E. Wijsman, E. Nemens, R.M. Garruto, L. Anderson, A. Andreadis, W.C. Wiederholt, M. Raskind, and G.D. Schellenberg. 1998. Tau is a

candidate gene for chromosome 17 frontotemporal dementia. *Annals Of Neurology*. 43:815-825.

Pozueta, J., R. Lefort, and M.L. Shelanski. 2012. Synaptic changes in Alzheimer's disease and its models. *Neuroscience*.

Qian, W., J. Shi, X. Yin, K. Iqbal, I. Grundke-Iqbal, C.-X. Gong, and F. Liu. 2010. PP2A regulates tau phosphorylation directly and also indirectly via activating GSK-3beta. *Journal Of Alzheimer's Disease: JAD*. 19:1221-1229.

Ramsden, M., L. Kotilinek, C. Forster, J. Paulson, E. McGowan, K. SantaCruz, A. Guimaraes, M. Yue, J. Lewis, G. Carlson, M. Hutton, and K.H. Ashe. 2005. Age-dependent neurofibrillary tangle formation, neuron loss, and memory impairment in a mouse model of human tauopathy (P301L). *J Neurosci*. 25:10637-10647.

Rendon, A., D. Jung, and V. Jancsik. 1990. Interaction of microtubules and microtubule-associated proteins (MAPs) with rat brain mitochondria. *The Biochemical Journal*. 269:555-556.

Rose, G.G. 1966. Zeiosis. I. Ejection of cell nuclei into zeiotic blebs. *Journal. Royal Microscopical Society (Great Britain)*. 86:87-102.

Ryoo, S.R., H.J. Cho, H.W. Lee, H.K. Jeong, C. Radnaabazar, Y.S. Kim, M.J. Kim, M.Y. Son, H. Seo, S.H. Chung, and W.J. Song. 2008. Dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A-mediated phosphorylation of amyloid precursor protein: evidence for a functional link between Down syndrome and Alzheimer's disease. *J Neurochem*. 104:1333-1344.

Salminen, A., A. Kauppinen, T. Suuronen, K. Kaarniranta, and J. Ojala. 2009. ER stress in Alzheimer's disease: a novel neuronal trigger for inflammation and Alzheimer's pathology. *J Neuroinflammation*. 6:41.

Saman, S., W. Kim, M. Raya, Y. Visnick, S. Miro, S. Saman, B. Jackson, A.C. McKee, V.E. Alvarez, N.C. Lee, and G.F. Hall. 2012. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J Biol Chem*. 287:3842-3849.

Santa-María, I., F. Hernández, C.P. Martín, J. Avila, and F.J. Moreno. 2004. Quinones facilitate the self-assembly of the phosphorylated tubulin binding region of tau into fibrillar polymers. *Biochemistry*. 43:2888-2897.

Santa-María, I., M.A. Smith, G. Perry, F. Hernández, J. Avila, and F.J. Moreno. 2005. Effect of quinones on microtubule polymerization: a link between oxidative stress and cytoskeletal alterations in Alzheimer's disease. *Biochimica Et Biophysica Acta*. 1740:472-480.

Santacruz, K., J. Lewis, T. Spires, J. Paulson, L. Kotilinek, M. Ingelsson, A. Guimaraes, M. DeTure, M. Ramsden, E. McGowan, C. Forster, M. Yue, J. Orne, C. Janus, A. Mariash, M. Kuskowski, B. Hyman, M. Hutton, and K.H. Ashe. 2005. Tau suppression in a neurodegenerative mouse model improves memory function. *Science (New York, N.Y.)*. 309:476-481.

Schenk, D., R. Barbour, W. Dunn, G. Gordon, H. Grajeda, T. Guido, K. Hu, J. Huang, K. Johnson-Wood, K. Khan, D. Kholodenko, M. Lee, Z. Liao, I. Lieberburg, R. Motter, L. Mutter, F. Soriano, G. Shopp, N. Vasquez, C. Vandevert, S. Walker, M. Wogulis, T. Yednock, D. Games, and P. Seubert. 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature*. 400:173-177.

Sengupta, A., M. Novak, I. Grundke-Iqbal, and K. Iqbal. 2006. Regulation of phosphorylation of tau by cyclin-dependent kinase 5 and glycogen synthase kinase-3 at substrate level. *FEBS Letters*. 580:5925-5933.

Sengupta, A., Q. Wu, I. Grundke-Iqbal, K. Iqbal, and T.J. Singh. 1997. Potentiation of GSK-3-catalyzed Alzheimer-like phosphorylation of human tau by cdk5. *Molecular And Cellular Biochemistry*. 167:99-105.

Sennvik, K., K. Boekhoorn, R. Lasrado, D. Terwel, S. Verhaeghe, H. Korr, C. Schmitz, T. Tomiyama, H. Mori, H. Krugers, M. Joels, G.J.A. Ramakers, P.J. Lucassen, and F. Van Leuven. 2007. Tau-4R suppresses proliferation and promotes neuronal differentiation in the hippocampus of tau knockin/knockout mice. *The FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology*. 21:2149-2161.

Serneels, L., J. Van Biervliet, K. Craessaerts, T. Dejaegere, K. Horré, T. Van Houtvin, H. Esselmann, S. Paul, M.K. Schäfer, O. Berezovska, B.T. Hyman, B. Sprangers, R. Sciot, L. Moons, M. Jucker, Z. Yang, P.C. May, E. Karran, J. Wiltfang, R. D'Hooge, and B. De Strooper. 2009. gamma-Secretase heterogeneity in the Aph1 subunit: relevance for Alzheimer's disease. *Science (New York, N.Y.)*. 324:639-642.

Seubert, P., T. Oltersdorf, M.G. Lee, R. Barbour, C. Blomquist, D.L. Davis, K. Bryant, L.C. Fritz, D. Galasko, and L.J. Thal. 1993. Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. *Nature*. 361:260-263.

Sharma, V.M., J.M. Litersky, K. Bhaskar, and G. Lee. 2007. Tau impacts on growth-factor-stimulated actin remodeling. *J Cell Sci*. 120:748-757.

Sherrington, R., E.I. Rogaev, Y. Liang, E.A. Rogaeva, G. Levesque, M. Ikeda, H. Chi, C. Lin, G. Li, and K. Holman. 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*. 375:754-760.

Shiryaev, N., Y. Jouroukhin, E. Giladi, E. Polyzoidou, N.C. Grigoriadis, H. Rosenmann, and I. Gozes. 2009. NAP protects memory, increases soluble tau and reduces tau hyperphosphorylation in a tauopathy model. *Neurobiology Of Disease*. 34:381-388.

Sigurdsson, E.M. 2009. Tau-focused immunotherapy for Alzheimer's disease and related tauopathies. *Current Alzheimer Research*. 6:446-450.

Simon, C.M., S. Sharif, R.P. Tan, and M.C. LaPlaca. 2009. Spinal cord contusion causes acute plasma membrane damage. *Journal of neurotrauma*. 26:563-574.

Singh, T.J., J.Z. Wang, M. Novak, E. Kontzekova, I. Grundke-Iqbal, and K. Iqbal. 1996. Calcium/calmodulin-dependent protein kinase II phosphorylates tau at Ser-262 but only partially inhibits its binding to microtubules. *FEBS Letters*. 387:145-148.

Smet-Nocca, C., M. Broncel, J.M. Wieruszeski, C. Tokarski, X. Hanoulle, A. Leroy, I. Landrieu, C. Rolando, G. Lippens, and C.P. Hackenberger. 2011. Identification of O-GlcNAc sites within peptides of the Tau protein and their impact on phosphorylation. *Molecular bioSystems*. 7:1420-1429.

Sonoda, Y., H. Mukai, K. Matsuo, M. Takahashi, Y. Ono, K. Maeda, H. Akiyama, and T. Kawamata. 2010. Accumulation of tumor-suppressor PTEN in Alzheimer neurofibrillary tangles. *Neuroscience Letters*. 471:20-24.

Sontag, E., V. Nunbhakdi-Craig, G. Lee, G.S. Bloom, and M.C. Mumby. 1996. Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. *Neuron*. 17:1201-1207.

Sontag, E., V. Nunbhakdi-Craig, G. Lee, R. Brandt, C. Kamibayashi, J. Kuret, C.L. White, M.C. Mumby, and G.S. Bloom. 1999. Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. *The Journal Of Biological Chemistry*. 274:25490-25498.

Spillantini, M.G., J.R. Murrell, M. Goedert, M.R. Farlow, A. Klug, and B. Ghetti. 1998. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 95:7737-7741.

Spittaels, K., C. Van den Haute, J. Van Dorpe, K. Bruynseels, K. Vandezande, I. Laenen, H. Geerts, M. Mercken, R. Sciot, A. Van Lommel, R. Loos, and F. Van Leuven. 1999. Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. *The American Journal Of Pathology*. 155:2153-2165.

Steinhilb, M.L., D. Dias-Santagata, T.A. Fulga, D.L. Felch, and M.B. Feany. 2007a. Tau phosphorylation sites work in concert to promote neurotoxicity in vivo. *Molecular Biology Of The Cell*. 18:5060-5068.

Steinhilb, M.L., D. Dias-Santagata, E.E. Mulkearns, J.M. Shulman, J. Biernat, E.-M. Mandelkow, and M.B. Feany. 2007b. S/P and T/P phosphorylation is critical for tau neurotoxicity in Drosophila. *Journal Of Neuroscience Research*. 85:1271-1278.

Stern, R.A., D.O. Riley, D.H. Daneshvar, C.J. Nowinski, R.C. Cantu, and A.C. McKee. 2011. Long-term consequences of repetitive brain trauma: chronic traumatic encephalopathy. *PM & R : the journal of injury, function, and rehabilitation*. 3:S460-467.

Strittmatter, W.J., A.M. Saunders, D. Schmechel, M. Pericak-Vance, J. Enghild, G.S. Salvesen, and A.D. Roses. 1993. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 90:1977-1981.

Sun, L., X. Wang, S. Liu, Q. Wang, J. Wang, M. Bennecib, C.-X. Gong, A. Sengupta, I. Grundke-Iqbal, and K. Iqbal. 2005. Bilateral injection of isoproterenol into hippocampus induces Alzheimer-like hyperphosphorylation of tau and spatial memory deficit in rat. *FEBS Letters*. 579:251-258.

Taguchi, H., S. Planque, Y. Nishiyama, J. Symersky, S. Boivin, P. Szabo, R.P. Friedland, P.A. Ramsland, A.B. Edmundson, M.E. Weksler, and S. Paul. 2008. Autoantibody-catalyzed hydrolysis of amyloid beta peptide. *The Journal Of Biological Chemistry*. 283:4714-4722.

Takashima, A. 2010. Tau Aggregation is a Therapeutic Target for Alzheimer's Disease. *Curr Alzheimer Res*.

Takeda, A., M.A. Smith, J. Avilá, A. Nunomura, S.L. Siedlak, X. Zhu, G. Perry, and L.M. Sayre. 2000. In Alzheimer's disease, heme oxygenase is coincident with

Alz50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. *Journal Of Neurochemistry*. 75:1234-1241.

Takei, Y., J. Teng, A. Harada, and N. Hirokawa. 2000. Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *The Journal Of Cell Biology*. 150:989-1000.

Tanemura, K., T. Akagi, M. Murayama, N. Kikuchi, O. Murayama, T. Hashikawa, Y. Yoshiike, J.M. Park, K. Matsuda, S. Nakao, X. Sun, S. Sato, H. Yamaguchi, and A. Takashima. 2001. Formation of filamentous tau aggregations in transgenic mice expressing V337M human tau. *Neurobiology Of Disease*. 8:1036-1045.

Tatebayashi, Y., T. Miyasaka, D.-H. Chui, T. Akagi, K.-i. Mishima, K. Iwasaki, M. Fujiwara, K. Tanemura, M. Murayama, K. Ishiguro, E. Planel, S. Sato, T. Hashikawa, and A. Takashima. 2002. Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 99:13896-13901.

Thal, L.J. 2006. Prevention of Alzheimer disease. *Alzheimer Dis Assoc Disord*. 20:S97-99.

Torres, C.R., and G.W. Hart. 1984. Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *The Journal Of Biological Chemistry*. 259:3308-3317.

Tremblay, M.A., C.M. Acker, and P. Davies. 2010. Tau phosphorylated at tyrosine 394 is found in Alzheimer's disease tangles and can be a product of the Abl-related kinase, Arg. *J Alzheimers Dis*. 19:721-733.

Trojanowski, J.Q., A.B. Smith, D. Huryn, and V.M.Y. Lee. 2005. Microtubule-stabilising drugs for therapy of Alzheimer's disease and other neurodegenerative disorders with axonal transport impairments. *Expert Opinion On Pharmacotherapy*. 6:683-686.

Tsukane, M., C. Yoshizaki, and T. Yamauchi. 2007. Development and specific induction of apoptosis of cultured cell models overexpressing human tau during neural differentiation: Implication in Alzheimer's disease. *Analytical Biochemistry*. 360:114-122.

Tucholski, J., J. Kuret, and G.V. Johnson. 1999. Tau is modified by tissue transglutaminase in situ: possible functional and metabolic effects of polyamination. *Journal Of Neurochemistry*. 73:1871-1880.

Turner, P.R., K. O'Connor, W.P. Tate, and W.C. Abraham. 2003. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Progress In Neurobiology*. 70:1-32.

Vandebroek, T., D. Terwel, T. Vanhelmont, M. Gysemans, C. Van Haesendonck, Y. Engelborghs, J. Winderickx, and F. Van Leuven. 2006. Microtubule binding and clustering of human Tau-4R and Tau-P301L proteins isolated from yeast deficient in orthologues of glycogen synthase kinase-3beta or cdk5. *The Journal Of Biological Chemistry*. 281:25388-25397.

Vandebroek, T., T. Vanhelmont, D. Terwel, P. Borghgraef, K. Lemaire, J. Snauwaert, S. Wera, F. Van Leuven, and J. Winderickx. 2005. Identification and isolation of a hyperphosphorylated, conformationally changed intermediate of human protein tau expressed in yeast. *Biochemistry*. 44:11466-11475.

Vassar, R., B.D. Bennett, S. Babu-Khan, S. Kahn, E.A. Mendiaz, P. Denis, D.B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M.A. Jarosinski, A.L. Biere, E. Curran, T. Burgess, J.C. Louis, F. Collins, J. Treanor, G. Rogers, and M. Citron. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science (New York, N.Y.)*. 286:735-741.

von Bergen, M., P. Friedhoff, J. Biernat, J. Heberle, E.M. Mandelkow, and E. Mandelkow. 2000. Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif ((306)VQIVYK(311)) forming beta structure. *Proceedings Of The National Academy Of Sciences Of The United States Of America*. 97:5129-5134.

Wang, H.-H., H.-L. Li, R. Liu, Y. Zhang, K. Liao, Q. Wang, J.-Z. Wang, and S.-J. Liu. 2010a. Tau overexpression inhibits cell apoptosis with the mechanisms involving multiple viability-related factors. *Journal Of Alzheimer's Disease: JAD*. 21:167-179.

Wang, J., and D.A. Richards. 2011. Spatial regulation of exocytic site and vesicle mobilization by the actin cytoskeleton. *PloS one*. 6:e29162.

Wang, J., Y.C. Tung, Y. Wang, X.T. Li, K. Iqbal, and I. Grundke-Iqbal. 2001. Hyperphosphorylation and accumulation of neurofilament proteins in Alzheimer disease brain and in okadaic acid-treated SY5Y cells. *FEBS Letters*. 507:81-87.

Wang, J.-Z., I. Grundke-Iqbal, and K. Iqbal. 2007. Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. *The European Journal Of Neuroscience*. 25:59-68.

Wang, J.Z., C.X. Gong, T. Zaidi, I. Grundke-Iqbal, and K. Iqbal. 1995. Dephosphorylation of Alzheimer paired helical filaments by protein phosphatase-2A and -2B. *The Journal Of Biological Chemistry*. 270:4854-4860.

Wang, J.Z., I. Grundke-Iqbal, and K. Iqbal. 1996. Glycosylation of microtubule-associated protein tau: an abnormal posttranslational modification in Alzheimer's disease. *Nature Medicine*. 2:871-875.

Wang, Y., L. Gao, S.W. Tse, and A. Andreadis. 2010b. Heterogeneous nuclear ribonucleoprotein E3 modestly activates splicing of tau exon 10 via its proximal downstream intron, a hotspot for frontotemporal dementia mutations. *Gene*. 451:23-31.

Weaver, C.L., M. Espinoza, Y. Kress, and P. Davies. 2000. Conformational change as one of the earliest alterations of tau in Alzheimer's disease. *Neurobiology Of Aging*. 21:719-727.

Wen, Y., E. Planel, M. Herman, H.Y. Figueroa, L. Wang, L. Liu, L.-F. Lau, W.H. Yu, and K.E. Duff. 2008. Interplay between cyclin-dependent kinase 5 and glycogen synthase kinase 3 beta mediated by neuregulin signaling leads to differential effects on tau phosphorylation and amyloid precursor protein processing. *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience*. 28:2624-2632.

Whiteman, I.T., O.L. Gervasio, K.M. Cullen, G.J. Guillemin, E.V. Jeong, P.K. Witting, S.T. Antao, L.S. Minamide, J.R. Bamburg, and C. Goldsbury. 2009. Activated actin-depolymerizing factor/cofilin sequesters phosphorylated microtubule-

associated protein during the assembly of alzheimer-like neuritic cytoskeletal striations. *J Neurosci*. 29:12994-13005.

Whiteman, I.T., L.S. Minamide, L. Goh de, J.R. Bamburg, and C. Goldsbury. 2011. Rapid changes in phospho-MAP/tau epitopes during neuronal stress: cofilin-actin rods primarily recruit microtubule binding domain epitopes. *PloS one*. 6:e20878.

Wilson, D.M., and L.I. Binder. 1995. Polymerization of microtubule-associated protein tau under near-physiological conditions. *The Journal Of Biological Chemistry*. 270:24306-24314.

Wittmann, C.W., M.F. Wszolek, J.M. Shulman, P.M. Salvaterra, J. Lewis, M. Hutton, and M.B. Feany. 2001. Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. *Science (New York, N.Y.)*. 293:711-714.

Xia, W. 2010. Exploring Alzheimer's disease in zebrafish. *Journal Of Alzheimer's Disease: JAD*. 20:981-990.

Xu, X., K.W. Chua, C.C. Chua, C.F. Liu, R.C. Hamdy, and B.H. Chua. 2010. Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury. *Brain Research*. 1355:189-194.

Yanagawa, H., S.H. Chung, Y. Ogawa, K. Sato, T. Shibata-Seki, J. Masai, and K. Ishiguro. 1998. Protein anatomy: C-tail region of human tau protein as a crucial structural element in Alzheimer's paired helical filament formation in vitro. *Biochemistry*. 37:1979-1988.

Zhang, W., J. Miao, J. Hao, Z. Li, J. Xu, R. Liu, F. Cao, R. Wang, J. Chen, and Z. Li. 2009. Protective effect of S14G-humanin against beta-amyloid induced LTP inhibition in mouse hippocampal slices. *Peptides*. 30:1197-1202.

Zhang, Y.J., Y.F. Xu, Y.H. Liu, J. Yin, H.L. Li, Q. Wang, and J.Z. Wang. 2006. Peroxynitrite induces Alzheimer-like tau modifications and accumulation in rat brain and its underlying mechanisms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 20:1431-1442.

Zmuda, J.F., and R.J. Rivas. 2000. Actin disruption alters the localization of tau in the growth cones of cerebellar granule neurons. *Journal Of Cell Science*. 113 (Pt 15):2797-2809.