THE ROLE OF OXIDATIVE STRESS ON TAU PROTEIN HOMEOSTASIS IN NEURODEGENERATIVE DISEASES

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Tauopathies are a class of neurodegenerative diseases consisting of the aggregation of the aberrant protein tau into paired helical filaments (PHF), which form neurofibrillary tangles (NFT), in the central nervous system. Tauopathies include Pick's disease, dementia pugilistica, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and Alzheimer's disease (AD). Recent studies show that the number of NFT directly correlates with the degree of dementia seen in AD [1].

In vivo phosphorylation of the tau protein is a hallmark of many neurodegenerative disorders including Alzheimer's disease (AD). Recent evidence has also demonstrated the cellular response to oxidative stress may play a role in tau pathogenesis. Here, two model systems were used to determine if a direct mechanistic link could be demonstrated between tau phosphorylation and the oxidative stress pathway. The first model system used was SH-SY5Y cells, a neuronal cultured cell line that endogenously expresses tau. In the second model system, wild-type tau and multiple tau variants were expressed in HEK cells. Cells were treated with hydrogen peroxide to induce the oxidative stress pathway, and changes in tau phosphorylation, tau kinases, and tau chaperones were monitored via western blot. Taken together, the results from these model systems suggest that a direct mechanistic link may exist between tau phosphorylation and the oxidative stress pathway.

Chapter 1

INTRODUCTION

1.1 Neurodegenerative Disease and The Tau Protein

1.1.1 Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder that slowly results in a gradual loss of cognitive function. AD is the most prevalent form of dementia; approximately 5.3 million Americans currently have AD, and it is the seventh leading cause of death [1]. The disease involves neuronal death defined by two biological markers of AD pathology: extracellular amyloid beta (Aβ) protein present as senile plaques (SP), and intracellular neurofibrillary tangles (NFT), consisting of hyperphosphorylated tau protein [2].

Despite a huge amount of research, the specific cause of the loss of cognitive function in AD remains unknown. It was originally believed that the presence of extracellular plaques was responsible, but there is poor correlation between the amount of plaque and cognitive loss [3]. Recent studies have shown that the increased number of NFT directly correlates with the degree of dementia seen in AD [4].

1.1.2 Tau Protein

Tau is a natively unfolded protein that associates with, stabilizes, and promotes polymerization of microtubules. Tau has 6 different isoforms, which are generated from a single gene by alternative splicing of exons 2, 3 and 10; these forms are shown in Figure 1.1 [5]. Tau is known to have 79 possible phosphorylation sites, which are regulated by kinases and phosphatases including glycogen synthase kinase-3β (GSK3β). Hyperphosphorylation of tau directly contributes to the diseased state, as its ability to bind and stabilize microtubules is

regulated by site-specific phosphorylation. Specifically, hyperphosphorylation shows decreased microtubule binding, which results in the self-assembly of NFT [4]. However, the causes of tau hyperphosphorylation are unknown. One factor that may be involved in causing this tau pathology is oxidative stress [6].

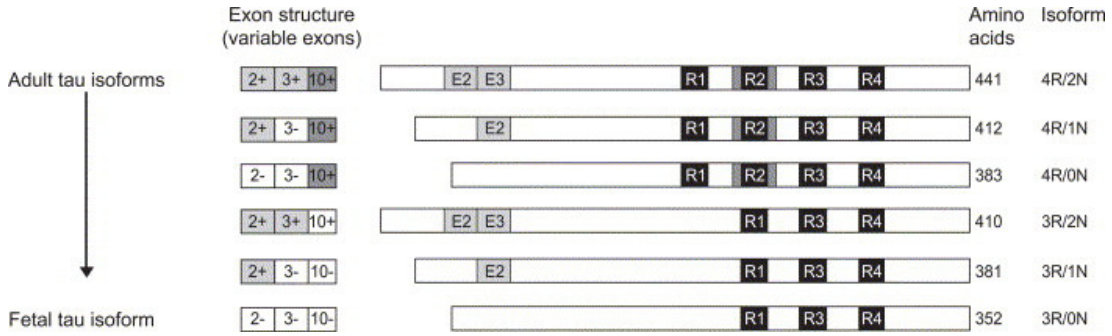


Figure 1.1 The six isoforms of tau [5]

1.1.3 Tau Related Chaperones

Along with the cellular stress response mechanism, cells have several quality control pathways. Cells regulate tau homeostasis, preventing the formation of NFT, through these pathways. Tau can either be degraded through the proteosomal pathway or returned to normal function through a refolding pathway. Major regulators in the refolding pathway are molecular chaperones. Chaperone proteins in cells ensure proteins are properly folded. Chaperones work cooperatively to regulate the function of proteins by manipulating ATPase activity of chaperone scaffolds, like heat shock protein 70 (Hsp70) and Hsp90 [7]. The basic chaperone-mediated folding pathway for most proteins begins after synthesis, with recognition by Hsp70, which then associates with Hsp90. The carboxy-terminus of Hsc70-interacting protein (CHIP) binds both Hsp70 and Hsp90. CHIP, an ubiquitin ligase, mitigates stress- related proteins after a cellular stress response, and is critical in the proteosomal degradation of abnormal tau [7].

The Hsp70 chaperone family was the first described chaperone regulator of tau. Two of its 13 family members are found in the cytoplasm, the constitutive Hsc70 protein, and

the inducible Hsp70 protein. It has been found that tau directly binds to Hsc70 [8]. Studies were able to connect microtubule dysfunction with chaperone-mediated regulation of tau, as well as conformational changes and hyperphosphorylation of tau [8]. Tau hyperphosphorylation disrupts refolding by chaperones, which may sequester tau in the cytosol, forming aggregates.

1.1.4 Tau Kinases

GSK3β, a known tau kinase that is also activated by the unfolded protein response (UPR), has been reported to be involved in the oxidative stress pathway [9]. Also, experimental studies have reported that GSK3β and cdk5 are acted on by reactive oxygen species (ROS) [10, 11]. These kinases may alter the phosphorylation of NF-E2-related factor 2 (Nrf2), which is a transcription factor controlling the expression of the antioxidant defense system. This would change the regulation of the oxidative stress response pathway. This may provide a potential mechanistic link between oxidative stress and tau biology.

1.2 Oxidative Stress and Tauopathies

1.2.1 Tauopathies

Tauopathies are a class of neurodegenerative diseases consisting of the aggregation and hyperphosphorylation of the aberrant protein tau into paired helical filaments (PHF), which form neurofibrillary tangles (NFT), in the central nervous system. In contrast to Aβ aggregation, which is specifically associated with AD, tau aggregates form in multiple neurodegenerative disorders, collectively termed tauopathies, including Pick's disease, dementia pugilistica, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and Alzheimer's disease (AD) [3, 5, 12].

Some tauopathies are genetic disorders caused by mutations in the tau gene, and at least 37 mutations have been identified which are associated with tauopathies. These mutations provide evidence that tau alterations alone can cause neurodegeneration. In

addition, these mutations increase the propensity of tau to aggregate and can be used to create model systems where tau is expressed recombinantly in cells or in transgenic mice [13-15].

1.2.2 Tau Mutations

It has been found that several mutations in tau give it a greater rate and level of phosphorylation, and a greater ability to sequester normal tau, as can be seen in Table 1. These tau mutations were found in a genetic neurodegenerative disease, FTPD-17. These mutations alone are enough to cause disease. It was also found that some mutated tau acquires a conformation that makes it a more favorable substrate for kinases, and aggregates at lower levels of phosphorylation [16]. These results illustrate that mutated tau more readily acquires toxic behavior induced by its abnormal hyperphosphorylation. However, pathways leading from mutations to neurodegeneration are unknown and oxidative stress may affect mutated tau differently.

Understanding the effects of oxidative stress pathways and mutations in tau will allow us to determine how loss of cellular control may lead to a disease state. Very few studies have been performed relating oxidative stress to tau homeostasis. Also, the approaches used by other studies relied on indirect methods, such as immunohistology and in vitro studies, or simply the upregulation of specific markers in the disease model [17-19]. Identification of altered gene function and pathways will provide the first step in developing therapeutic strategies.

Table 1.1 Tau Mutations

Mutation	Type	Effect	Reference
Δ280K	Missense	Aggregation, decreased microtubule affinity, neuronal loss	[14]
P301L	Substitution	Neuronal impairment, NFT formation	[14]
N296N	Silent	Greater 4R to 3R ratio (splicing)	[20]
VPR Triple Mutant (R406W, P301L, V337M)	Substitution	Hyperphosphorylation, aggregation, decreased microtubule affinity	[21, 22]

1.2.3 Oxidative Stress

Cells maintain a balanced reducing environment through cytoprotective pathways. Major components of these pathways are enzymes that reduce or inactivate ROS. Oxidative stress results from disturbances in this normal reduction/oxidation state caused by the production and accumulation of ROS that damage cellular components. Antioxidant-response element (ARE) controls the expression of antioxidant genes. NF-E2-related factor 2 (Nrf2), a transcription factor, becomes activated by an increase in ROS, and binds to the ARE and regulates ARE-mediated antioxidant enzyme gene expression and induction in response to oxidative stress.

Neurons are more easily subjected to oxidative stress because of their unique conditions, including high energy and oxygen consumption rate of the brain, high levels of transition metals, and lower levels of antioxidants relative to other organs [6]. Therefore it is particularly difficult to balance the production of ROS and antioxidant defenses in neurons. Chronic oxidative stress can occur in neurons, which is suspected to play a role in neurodegenerative diseases.

Other than hyperphosphorylation, oxidative stress may be involved in protein misfolding, which can alter microtubule stabilization by tau. Also, the levels of chaperones vary during normal cellular aging, which have been implicated in neurodegeneration, and may be affected by oxidative stressors [23].

Emerging evidence shows that oxidative stress may play a role in tau pathology; however, it is unclear how oxidative stress relates to tau regulation pathways. Many immunohistological studies demonstrate that an increase of ROS correlates with neurodegeneration. One recent study linked the prevention of glutathione synthesis, an antioxidant pathway, to increased tau phosphorylation in neuroblastoma cells [17]. Also the addition of oxidative stressors such as tert-butylhydroperoxide (tBHP) and hydrogen peroxide (H_2O_2) in SH-SY5Y neuroblastoma cell lines caused cell cytotoxicity, tau dephosphorylation,

and caspase-3 activation, which leads to apoptosis [18, 19]. These studies indicate a plausible mechanistic link between tau phosphorylation levels and the cellular redox state.

The oxidative stress mechanism of the cell may explain crucial aspects such as the timing, selective cellular vulnerability and progression of tauopathies. Oxidative stress occurs in the vast majority of, if not all, degenerative diseases of the nervous system [24]. A better understanding of the role of oxidation may serve to define damaged metabolic networks at early stages of disease and to advance therapeutic interventions to attenuate disease progression.

1.2.4 ROS Pathway Effects on Tau

Studies show that an increase in ROS correlates with neurodegenerative disorders; however, the causative roles of oxidative stress in these disorders are not well understood. The overall objective of this work was to determine whether oxidative stress precedes tau pathology. To do this, we tested the importance of several oxidative stress mechanisms by which tau could be affected, as shown in Figure 1.2. We also determined how expression of various tau mutants may impact this stress.

There are three hypothesized pathways in which ROS may affect tau. Preliminary results focus on the first pathway, direct oxidation. By directly stressing the cells by adding ROS, and following changes in protein expression levels, I will be able to verify a relationship between oxidative stress and tau pathology. The second pathway involves the oxidative stress response pathway that affects chaperone levels. It is known that ROS activate Nrf2, which regulates the ARE, thereby affecting chaperone levels. The last pathway involves the activation of kinases by ROS, thus indirectly activating tau.

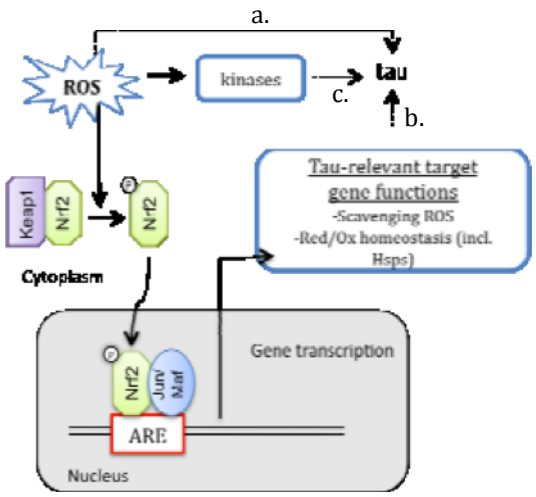


Figure 1.2: Possible pathways ROS effects on tau

Chapter 2

OXIDATIVE STRESS AND THE PROTEIN TAU

This chapter examines the relationship between the *in vivo* phosphorylation of tau, which is associated with multiple neurodegenerative disorders, and the oxidative stress pathway. Both oxidative stress activation and the presence of hyperphosphorylated tau are observed in Alzheimer's disease, as described in Chapter 1. The goal of the work presented here is to determine if a direct, mechanistic link can be demonstrated between the two events.

The experimental approach used two different cell culture models. To determine how oxidative stress would impact tau biology, the oxidative stress pathway was activated with chemical reagents in SH-SY5Y cells, which endogenously express tau, and HEK293 cells. Changes in tau expression levels and phosphorylation state were measured. Both wild-type tau and tau variants were recombinantly overexpressed in HEK cells, which do not endogenously express tau, and cell samples were assayed for oxidative stress activation.

2.1 Materials and Methods

2.1.1 Materials

Where not otherwise specified, all reagents and buffer components were obtained from major chemical suppliers. Cell culture reagents were obtained from Invitrogen.

2.1.2 Cell Culture

2.1.2.1 SH-SY5Y Cells

SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in a 1:1 mixture of Eagles's Minimum Essential Medium (EMEM) and Ham's F12 Medium supplemented with 10% fetal bovine serum (FBS), 50

U/mL penicillin and 50 μg/mL streptomycin at 37° C and 5% CO_2. Media was replenished every 2-3 days. Cells were maintained in T-75 flasks and passaged at a ratio of 1:20 to 1:50 when flasks reached 80-90% confluency.

SH-SY5Y cells were differentiated with retinoic acid for all experiments. Cells were removed from the T-75 flasks with 0.25% trypsin, 0.5 mM EDTA, and the cell concentration was determined by hemocytometry. Cells were plated in 6-well plates at a density of $5x10^4$ cells/well. Approximately 5-7 days after plating, media was supplemented with 10 μM retinoic acid. Cells were cultured in the presence of retinoic acid for a minimum of 8 days prior to use in oxidative stress experiments.

2.1.2.2 T-REx-293 Cells

Human embryonic kidney cells (HEK293) stably transfected with a plasmid containing the tetracycline (Tet) repressor were obtained from Invitrogen and are referred to by the commercial name, T-REx-293. T-REx-293 cells were cultured in Dulbecco's modification of Eagles's Minimum Essential Medium (DMEM) with 4mM L-glutamine and high glucose supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin at 37° C and 5% CO_2. Media was replenished every 2-3 days. Media also included 5 μg/mL blasticidin, the selective marker for the Tet repressor plasmid. Cells were maintained in T-75 flasks and passaged at a ratio of 1:5 to 1:8 when flasks reached 80-90% confluency.

2.1.2.3 Creation of Tau Variants

Stratagene's QuikChange II XL Site-Directed Mutagenesis Kit was used for introducing mutations into the tau gene. Primers were designed using the Stratagene primer design program. DNA was sequenced by the University of Delaware Sequencing and Genotyping Center, and compared to the expected sequence using the Sequencher program.

The tau variants were transformed into the pDONR vector, using ccdB in survival chemically competent cells. This recombination reaction results in the entry clone. The entry clone was then combined with the destination vector, pDEST31, to produce the final

expression clone. Plasmids were digested and run on a gel for confirmation. Cultures were amplified for mammalian transfection using the Promega Maxiprep kit.

2.1.2.4 Cellular Transfection

T-Rex-293 cells were transfected with the pT-REx-DEST31 Gateway vector (Invitrogen) containing the gene for wild-type tau (4R isoform), tau P301L, Δ280K, N296N, VPR triple mutant, or a control gene (lacZ) using the reagent Lipofectamine 2000 (Invitrogen). Some cells were also treated with Lipofectamine in the absence of DNA as a negative control for transfection.

For transfection, T-REx-293 cells were plated in 6-well plates at a density of $1x10^5$ cells/well and allowed to grow for 48 hours until approximately 50% confluent. Approximately 6 hours prior to transfection, media was changed to DMEM without antibiotics.

To prepare the DNA/Lipofectamine complexes, for each transfection well, 0.5 μg of plasmid DNA were diluted in 250 μL of Opti-MEM media (Invitrogen) and 1 μL of Lipofectamine 2000 was separately diluted in 250 μL of Opti-MEM. Following a 5 minute incubation at room temperature, the DNA and Lipofectamine solutions were mixed and incubated for an additional 20 minutes at room temperature before being added to the cells. Cells were allowed to grow in antibiotic-free media for a 48-hour recovery period after transfection before switching to selective media including 5 μg/mL blasticidin and geneticin in different concentrations (0, 200, 500 and 800 μg/mL). After approximately 7 days of selection, non-transfected control cells grown with 500 and 800 μL/mL geneticin concentrations were no longer viable. Stably transfected cells were then propagated in 500 μg/mL geneticin.

2.1.2.5 Development of a Stable Cell Line

In order to create a stable cell line for constant tau expression, colonies of T-REx-293 cells were grown that were transfected with either wild-type tau, or the mutations listed in

Table 1. Cells were plated in 6-well plates at a low enough density to form individual colonies. Several colonies were selected using clonal discs, and then grown in separate 6-well plates. Tau expression was induced using tetracycline. Western blotting was used to determine the level of tau expression in individual clones. Colonies expressing a high level of tau were picked for future oxidative stress experiments.

2.1.2.6 Chemical Induction of the Oxidative Stress Pathways

To determine the effect of oxidative stress on tau biology, oxidative stress was induced using chemical reagents in differentiated SH-SY5Y cells, and transfected T-REx-293 cells. To induce oxidative stress, media containing 50 nM hydrogen peroxide was added to cells in 6-well plates. As a positive control for tau phosphorylation, some cells were also treated with 75 nM okadaic acid, a phosphatase inhibitor. At specified times after treatment, cell samples were taken and analyzed for both oxidative stress activity and changes in tau phosphorylation state.

2.1.3 Recombinant Protein Expression

T-REx-293 cells stably transfected with the DEST31 plasmid containing the gene of interest as well as non-transfected control cells were plated in 6-well plates at a density of 2 x 10^5 cells/well. Cells were grown in selective media for 24 hours before protein expression was induced by addition of 1 μg/mL tetracycline. At specified times after treatment, cell samples were taken for analysis by Western blotting.

2.1.4 Western Blotting

For analysis of protein levels by Western blotting, cells were lysed in M- Per Protein Extraction Reagent including Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Pierce). Insoluble cellular debris was removed by centrifugation at 12,000 x g for 15 minutes. Total protein concentration in cleared lysates was quantified using the BCA assay (Pierce) with BSA as a standard. Equal amounts of protein for each sample

(typically 6.5 μg) were run on 4-20% SDS gels for 2 hours at 120 V. Gels were then transferred to nitrocellulose overnight (>16 hours) at 10 V. Nitrocellulose membranes were blocked for >2 hours with a blocking solution of either powdered milk or bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween (TBST). Membranes were then incubated with primary antibody overnight at 4 °C. The specific supplier, blocking conditions, incubation conditions and secondary antibody for each primary antibody used in this work are listed in Table 2. After three 10 minutes washes with TBST, membranes were incubated with an HRP-conjugated secondary antibody (GE Healthcare) and the blots were visualized using the ECL-Plus kit (GE Healthcare) on a Typoon 9500 scanner, and quantified with ImageQuant software (GE Healthcare).

Table 2.1 Primary Antibodies

Antigen	Supplier	Catalogue #	Dilution	Secondary Antibody	Blocking Solution	Incubation Solution
GAPDH	Invitrogen	39-8600	1:15000	Mouse	5% Milk	1% BSA
Tau	Biosource	AHB0042	1:1000	Mouse	5% Milk	3% BSA
HO-1	Stressgen	OSA-150	1:1000	Rabbit	5% Milk	3% BSA
Phospho Tau	Invitrogen	44768G	1:1000	Rabbit	5% BSA	3% BSA
GSK3B	Thermo Scientific	MA1-7621	1:1000	Mouse	5% Milk	3% BSA
Phospho GSK3B	Thermo Scientific	PA1-4688	1:1000	Rabbit	5% Milk	3% BSA
HSP70	Thermo Scientific	MA3-008	1:2500	Mouse	5% Milk	3% BSA

2.1.5 Quantification and Statistical Analysis

Western blots and DNA gels were quantified using ImageQuant software (GE Healthcare), and statistical analysis was performed using Minitab software. Regression analysis with a 95% confidence interval was used between the HO-1 oxidative stress data and each antibody measured. A line was fit between the data to determine if a linear relationship existed (within this 95% confidence). A two-way analysis of variance (ANOVA) test with a 95% confidence interval was then used to determine if a statistically significant difference

existed among the samples versus treatment and time, for each antibody measured. This analysis was performed for wild type tau and each variant.

2.2 Results

2.2.1 Oxidative Stress of SH-SY5Y Cells

2.2.1.1 SH-SY5Y Cells

Human neuroblastoma SH-SY5Y cells were used as a model system as they natively express tau. In an undifferentiated state, these cells can be grown continuously in culture and have an epithelial-like morphology. By adding 10uM retinoic acid, SH-SY5Y cells differentiate into a neuronal-like morphology (Figure 2.1).

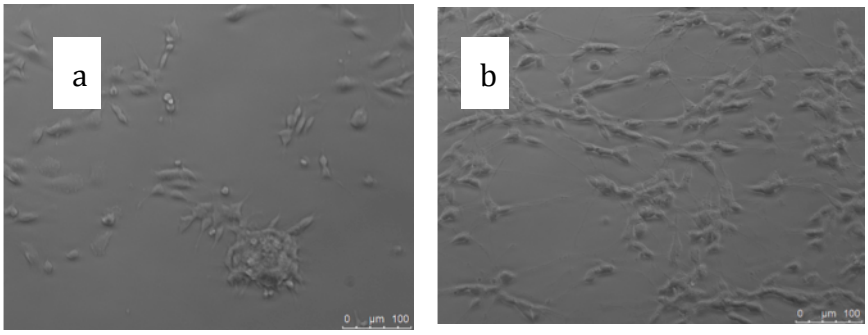


Figure 2.1 SH-SY5Y cells a) undifferentiated and b) after 3 days in retinoic acid

2.2.1.2 Direct Oxidation of Tau in SH-SY5Y Cells

To determine how oxidative stress impacted tau biology, the oxidative stress pathway was induced by adding hydrogen peroxide (H_2O_2) to SH-SY5Y cells. Okadaic acid (OA), a phosphatase inhibitor, was dissolved in DMSO and used as a positive control for tau phosphorylation. These treatments have been used in several oxidative stress studies [17, 19, 25]. Dimethyl sulfoxide (DMSO) treatment alone was used as a negative control.

To confirm that the oxidative stress pathway was activated, I monitored the regulation of heme oxygenase 1 (HO-1), an inducible stress protein, which confers cytoprotection against oxidative stress *in vitro* and *in vivo* [26, 27]. Total tau protein levels were measured using the Tau5 antibody. A phosphospecific tau antibody was also used, which

recognizes tau phosphorylated at serine 199 or 200, as hyperphosphorylation at these sites is associated with increased propensity to form tangles [28].

Time points were taken at 1 and 3 hours. Western blots are shown in Figures 2.2, and quantification is shown in Figure 2.3. As expected, addition of H_2O_2 induced HO-1 expression, as indicated in Figure 2.3b. Also, OA treatment increased tau phosphorylation at 1 hour. However, there was large variability in the results. More experiments at multiple time points need to be performed in order to make any further conclusions.

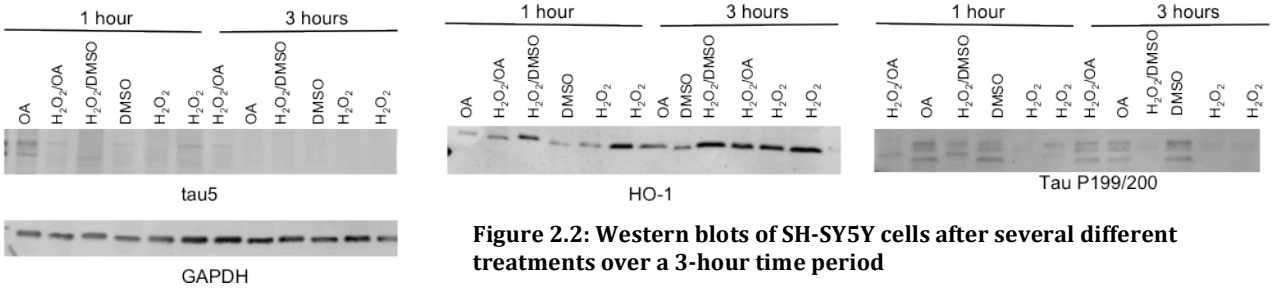


Figure 2.2: Western blots of SH-SY5Y cells after several different treatments over a 3-hour time period

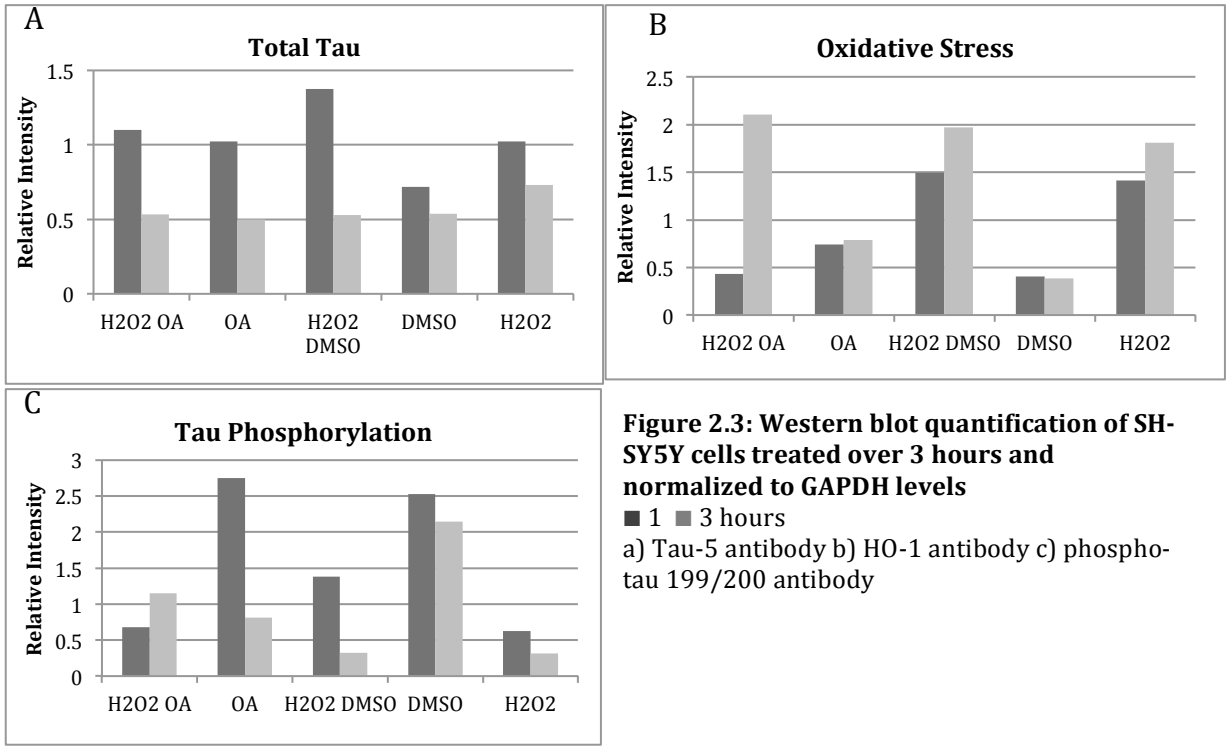


Figure 2.3: Western blot quantification of SH-SY5Y cells treated over 3 hours and normalized to GAPDH levels

■ 1 ■ 3 hours

a) Tau-5 antibody b) HO-1 antibody c) phospho-tau 199/200 antibody

2.2.2 Inducible Overexpression of Wild Type and Mutated Tau

Mutagenesis was performed on tau, in a donor vector, to incorporate the mutations listed in Table 1. The donor vector was then recombined into an expression vector. T-REx-293 cells were transfected with the expression vector containing the genes for either wild type tau, or the tau mutations.

Displayed in Figure 2.4 is a Western blot confirming these cell lines express tau only under control of the tetracycline-induced promoter. Tau was observed in the wild type sample when induced, but not in the uninduced or non-transfected control samples. Two bands appear on the Western blots, which can be attributed to degrees of phosphorylation [14].

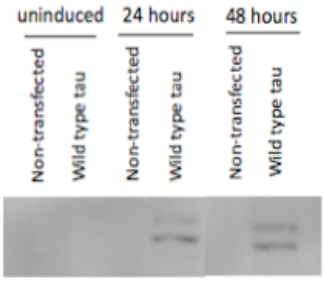


Figure 2.4 Expression of tetracycline-induced tau confirmed by Western blot

2.2.3 Development of a Stable Cell Line

In order to create a stable cell line for constant tau expression, colonies of T-REx-293 cells were grown that were transfected with either wild-type tau, or the mutations listed in Table 1. Tau expression was induced using tetracycline, and several colonies were selected for Western blotting to verify transfection and level of tau expression. Figure 2.5 displays different levels of tau expression in eight different colonies selected. GPADH was also assayed as a protein loading control. This demonstrates stable cell line development was necessary due to the different levels of expression each clone has. Colonies expressing a high level of tau were picked for future oxidative stress experiments.

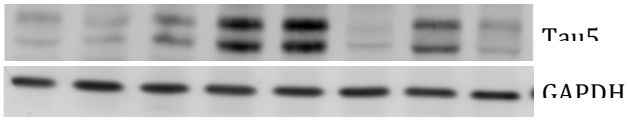


Figure 2.5 Western blot displaying 8 WT tau colonies selected, with different expression levels

2.2.4 Oxidative Stress of T-REx-293 Cells

T-REx-293 single clones were used to determine how oxidative stress impacted tau biology. The oxidative stress pathway was induced by adding hydrogen peroxide (H_2O_2), as outlined above, to cells transfected with wild type tau, and the variants listed in Table 1. Similar to the SH-SY5Y cells, T-REx-293 cells were treated with OA dissolved in DMSO to inhibit phosphorylation, and DMSO as a negative control.

To confirm that the oxidative stress pathway was activated, I monitored the regulation of heme oxygenase 1 (HO-1). Total tau protein levels were measured using the Tau5 antibody. A phosphospecific tau antibody was also used, which recognizes tau phosphorylated at serine 199 or 200. Antibodies for GSK3β, a known tau kinase, and GSK3β-serine-9, were also used. Phosphorylation of GSK3β at this site inhibits kinase activity; thus, an increase in phosphorylation would decrease activity. Finally, an antibody for HSP70, a known tau chaperone, was measured.

2.2.4.1 Wild Type Tau

Results for wild-type tau are shown in Figures 2.6, 2.7, and 2.8 below. Time points were taken at 1, 3, 6, 12, and 24 hours. Total tau levels had no significant trend throughout each time period for each different treatment. For the oxidative stress experiment, the HO-1 antibody increased in treatments involving H_2O_2, and H_2O_2 with DMSO, indicating the oxidative stress pathway is active; however, HO-1 levels stayed constant throughout the combined treatment with H_2O_2 and OA. Therefore, we can theorize that OA affects the oxidative stress of H_2O_2. Levels of HO-1 for the DMSO control and OA treatments also stayed constant. As shown in Figure 2.7c, phosphorylation levels of tau increased after 6 hours of H_2O_2 treatment, while no phosphorylation of tau under the OA, DMSO, or H_2O_2/OA treatments was seen. Large variability has been observed previously in OA-induced phosphorylation among different tau sites; therefore, it is not surprising that we see no increase in phosphorylation levels of only sites 199 and 200 [29].

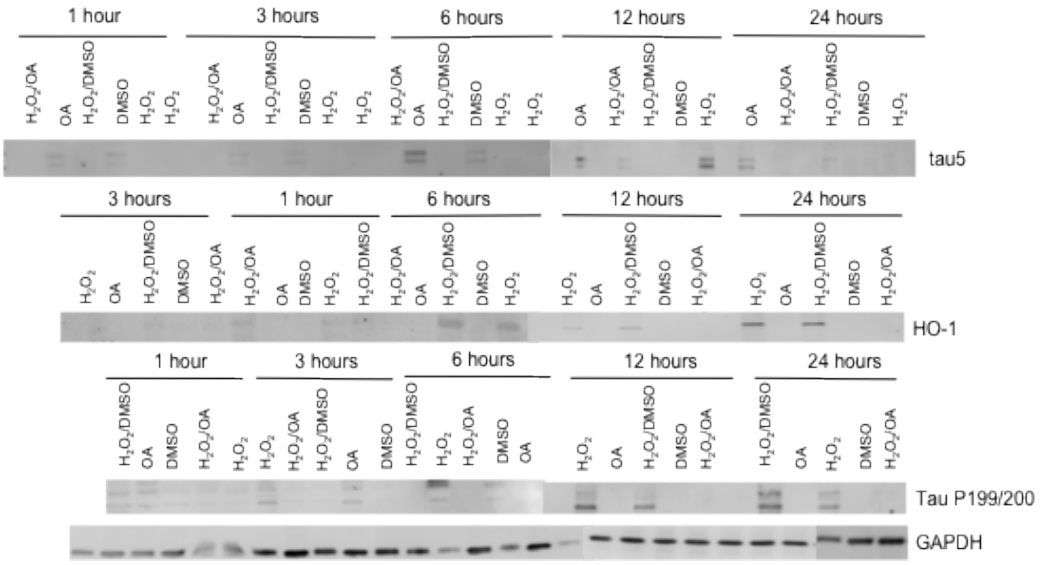


Figure 2.6 Western blot of T-REx-293 cells after several different treatments over 24 hours

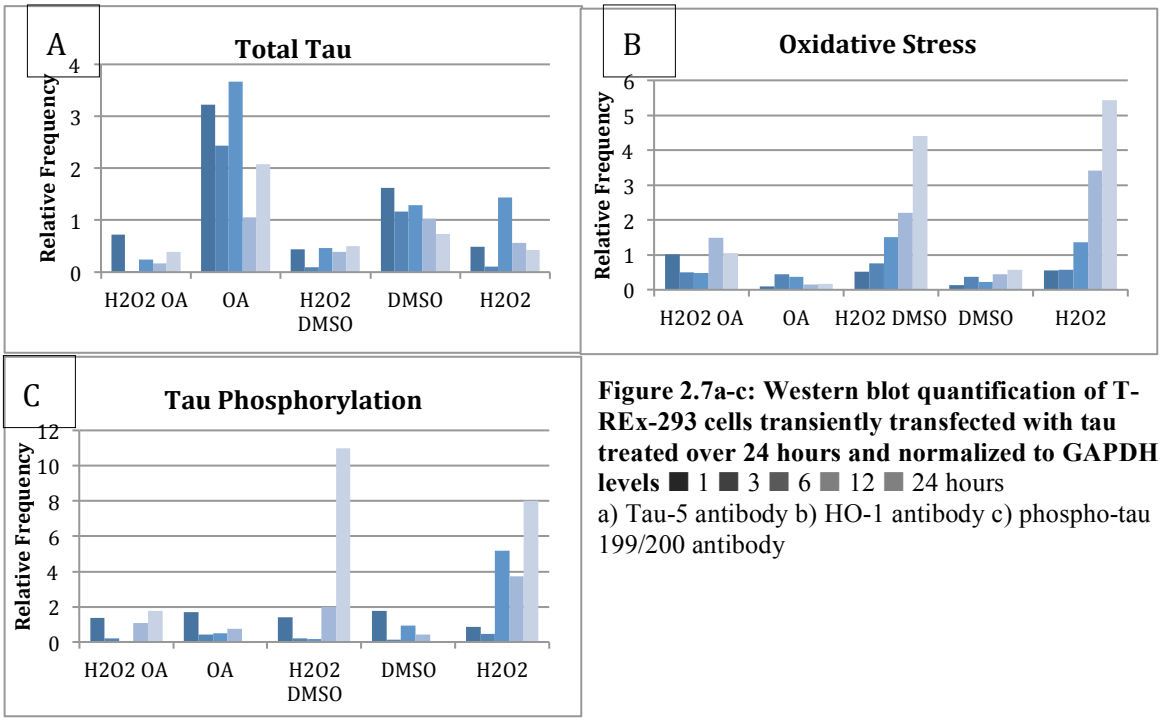


Figure 2.7a-c: Western blot quantification of T-REx-293 cells transiently transfected with tau treated over 24 hours and normalized to GAPDH levels ■ 1 ■ 3 ■ 6 ■ 12 ■ 24 hours
a) Tau-5 antibody b) HO-1 antibody c) phospho-tau 199/200 antibody

Similar results were also seen for the tau kinase, GSK3β (Figure 2.8). As oxidative stress increases, the tau kinase levels are also increased, in a time independent manner. This significantly correlates with the increase in tau phosphorylation seen in the previous Figure 2.7c. Inactive tau kinase levels also decreased with oxidative stress. Hsp70, a Tau chaperone, was also measured. And these levels also time-independently increased under the oxidative stress treatments (Figure 2.8c).

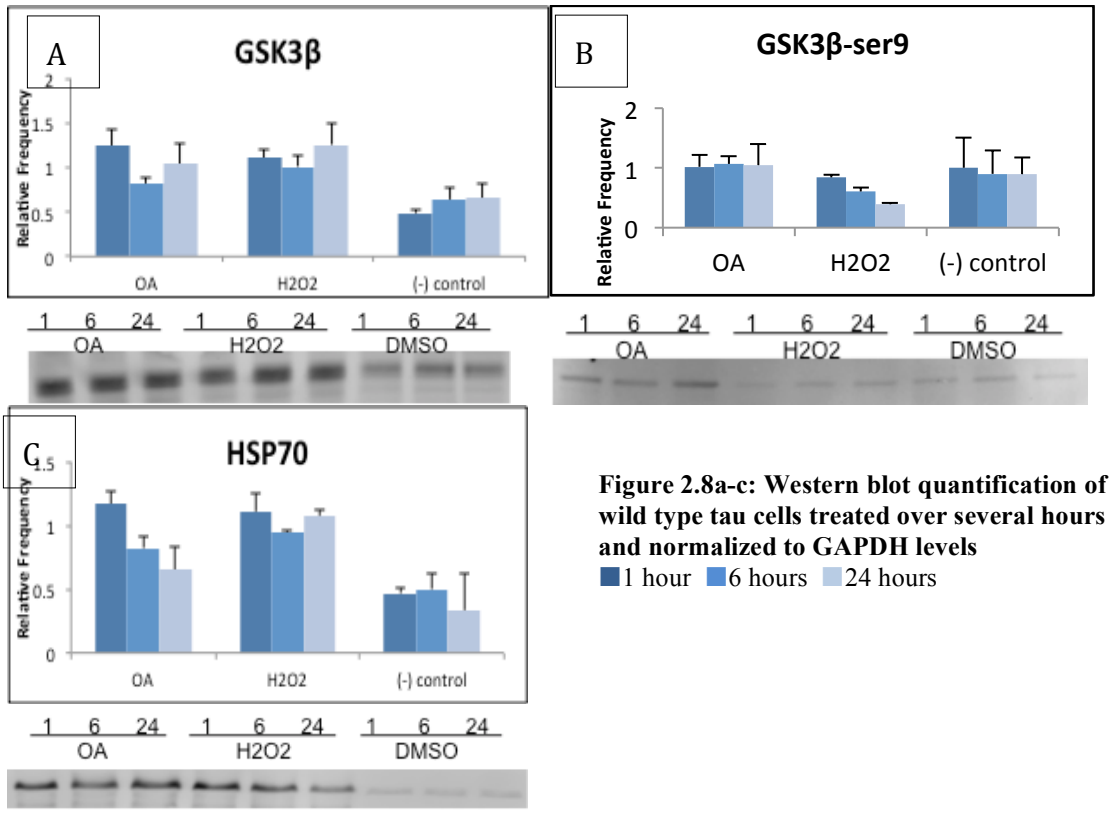
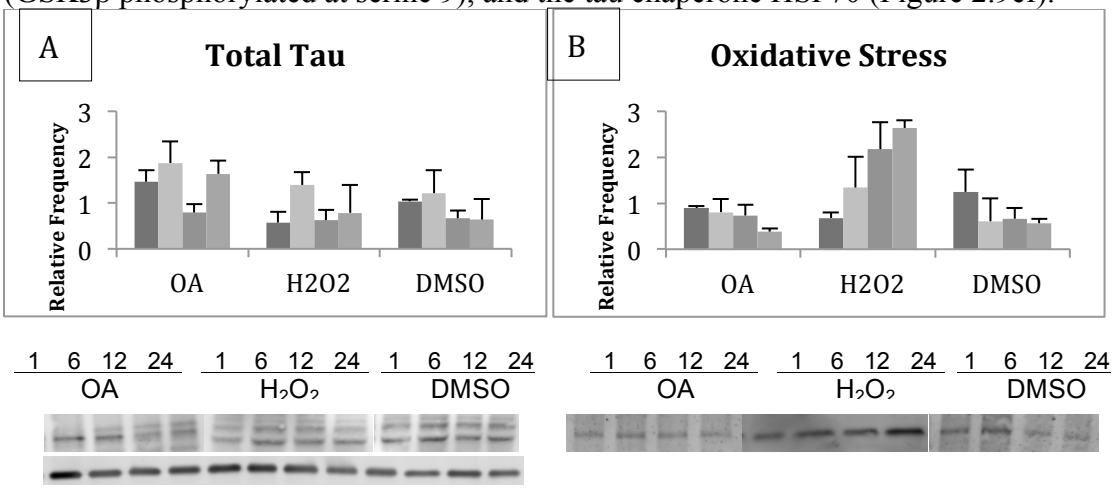


Figure 2.8a-c: Western blot quantification of wild type tau cells treated over several hours and normalized to GAPDH levels
1 hour 6 hours 24 hours

2.2.4.2 N296N Variant

As expected, tau phosphorylation increased along with the tau kinase GSK3β in the N296N variant under oxidative stress (Figure 2.9cd). However, unlike WT tau, the change in GSK3β levels was not time independent. Also, no change was seen in inactive GSK3β (GSK3β phosphorylated at serine 9), and the tau chaperone HSP70 (Figure 2.9ef).



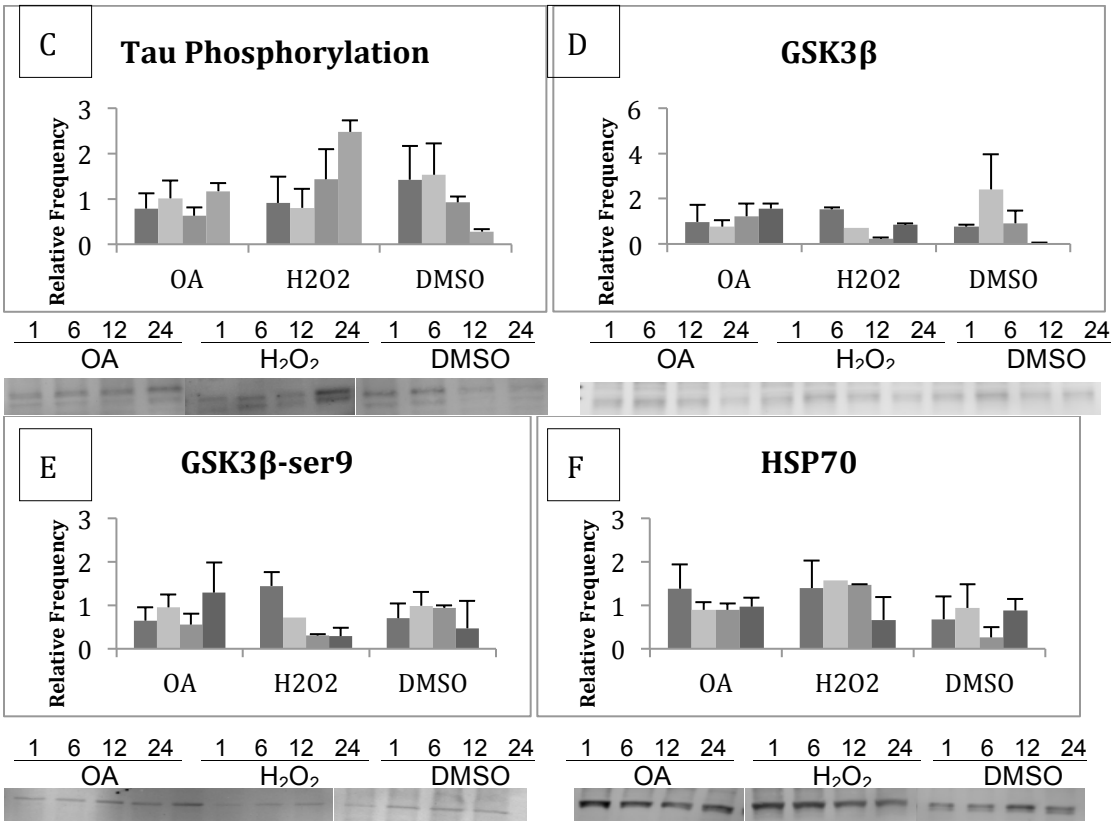
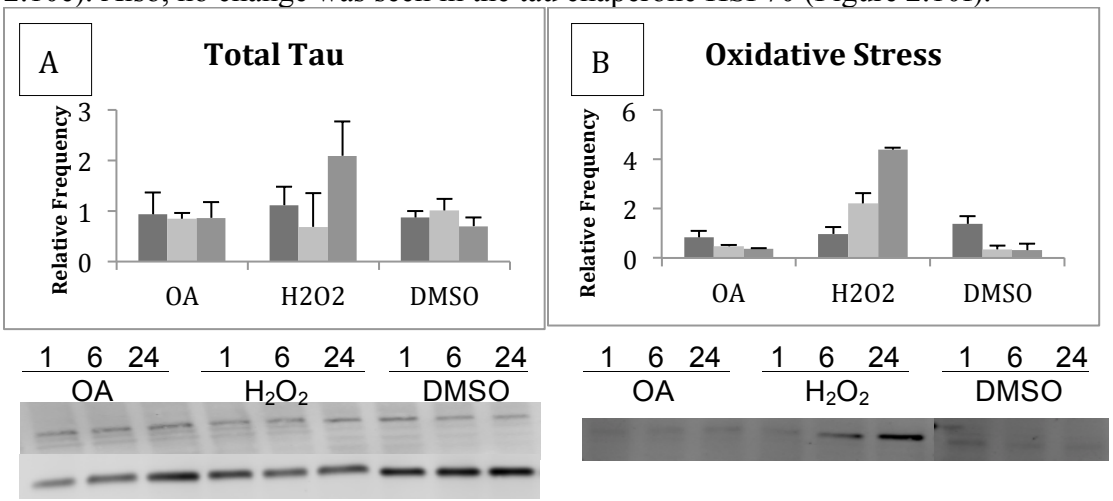


Figure 2.9a-f: Western blot quantification of N296N tau variant cells treated over several hours and normalized to GAPDH levels. 1 hour 6 hours 12 hours 24 hours

2.2.4.3 VPR Triple Mutant

Along with WT tau, tau phosphorylation increased along with a time-independent increase of the tau kinase GSK3β in the VPR triple mutant variant under oxidative stress (d). However, unlike WT tau, inactive GSK3β decreased in a time-independent manner (Figure 2.10e). Also, no change was seen in the tau chaperone HSP70 (Figure 2.10f).



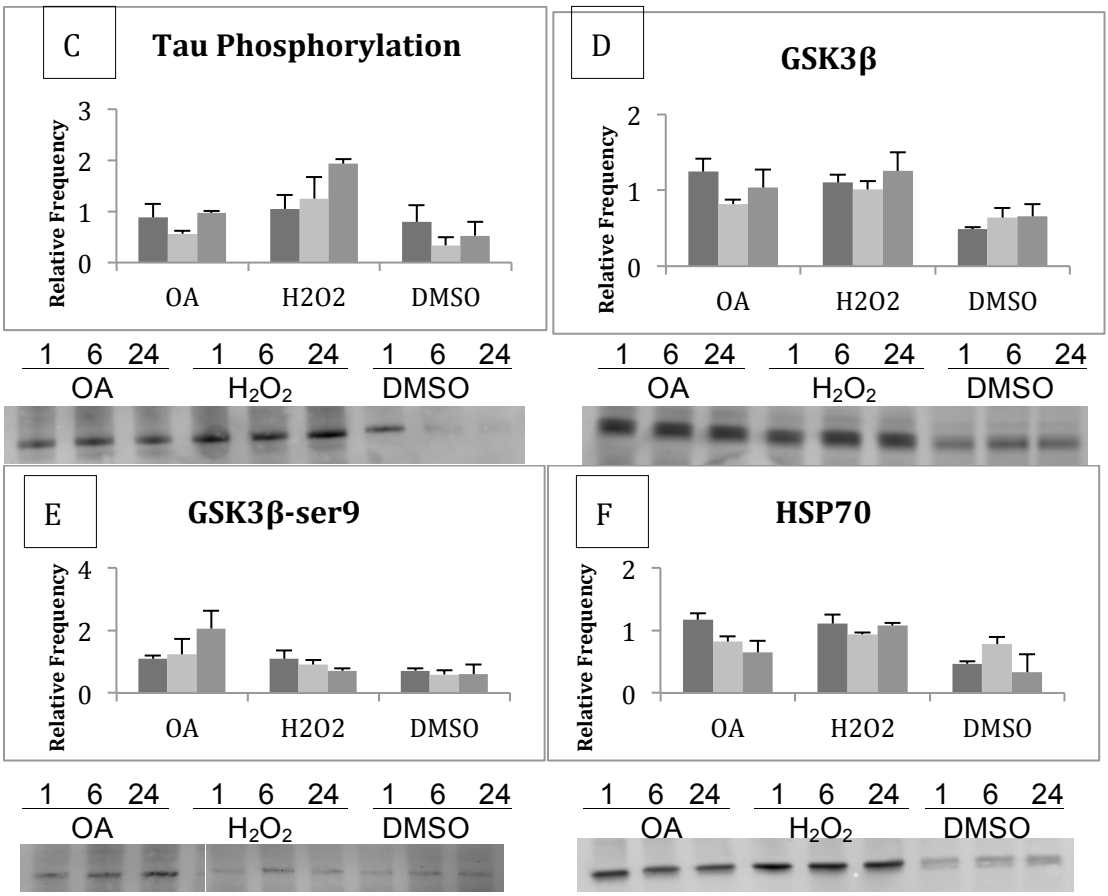


Figure 2.10a-f: Western blot quantification of VPR tau variant cells treated over several hours and normalized to GAPDH levels. 1 hour 6 hours 24 hours

2.2.4.4 P301L Variant

The P301L variant had the most similar results to WT tau compared to the other tau variants. Tau phosphorylation increased, along with time independent increases in GSK3β and HSP70, while inactive GSK3β decreased under oxidative stress (Figure 2.11c-f).

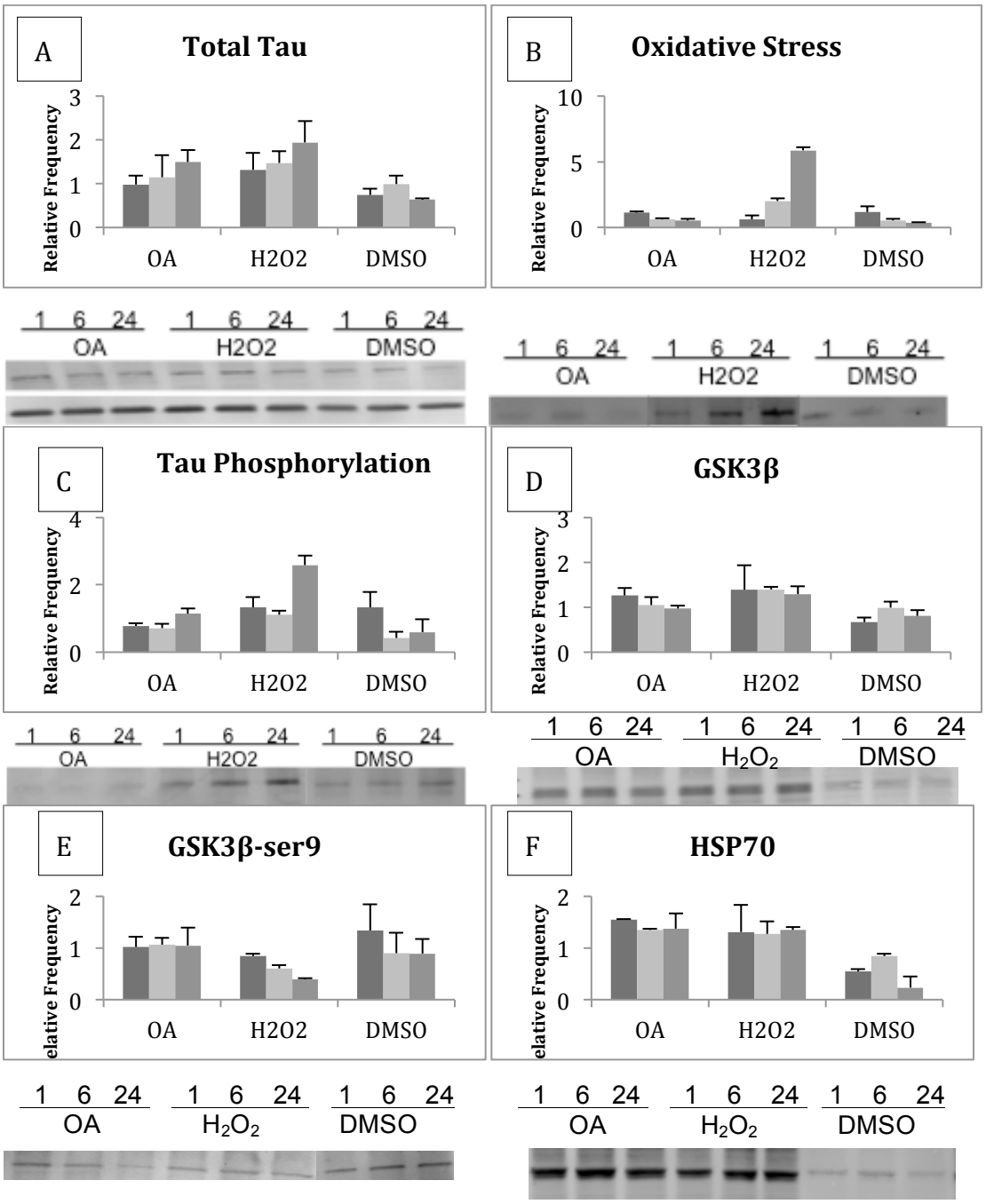


Figure 2.11a-f: Western blot quantification of P301L tau variant cells treated over several hours and normalized to GAPDH levels. ■ 1 hour ■ 6 hours ■ 24 hours

2.2.4.5 Δ280K Variant

The Δ280K variant was the only variant with no time independent changes. Phosphorylated tau along with GSK3β and HSP70 increased, as inactive GSK3β decreased under oxidative stress (Figure 2.12 c-f).

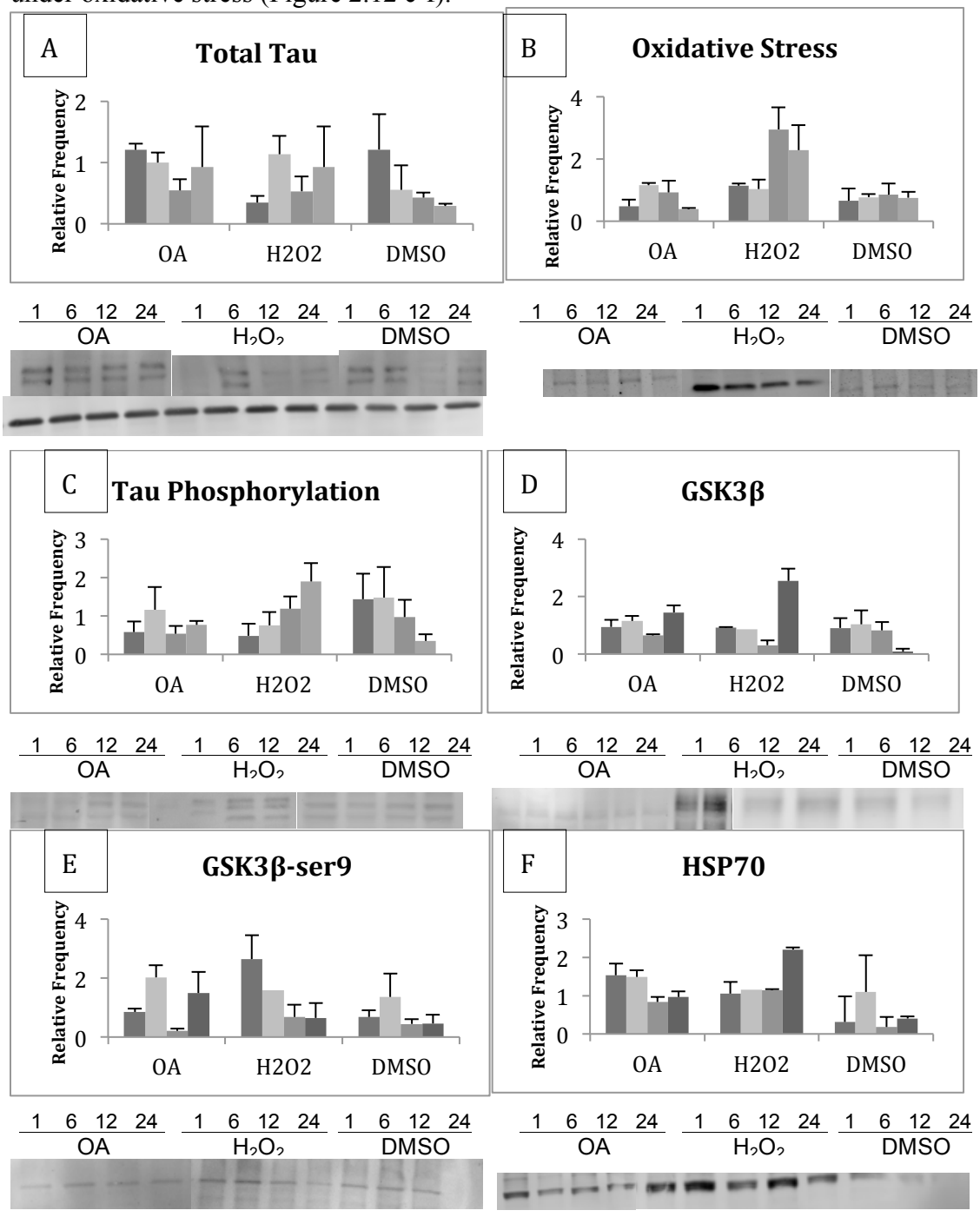


Figure 2.12a-f: Western blot quantification of Δ280K tau variant cells treated over several hours and normalized to GAPDH levels. 1 hour 6 hours 12 hours 24 hours

Overall, varied results were seen in between each variant case. Only WT tau and P301L tau had similar changes for each antibody measured. The only similarity throughout each variant was the increase of phosphorylated tau under oxidative stress. GSK3β also increased for each variant; however, the change was time-independent for WT, triple mutant, and P301L tau variants.

Inactive GSK3β and HSP70 had the most variability across the tau variants. Inactive GSK3β decreased for WT, Δ280K, and P301L tau variants. It also decreased for the triple mutant in a time independent manner, and there was no change for the N296N variant. For the HSP70 antibody, there was an increase seen in the Δ280K variant, and a time independent increase seen for the WT and P301L variants. However, no change was seen for the triple mutant or N296N variants. These results are summarized in Table 3 below.

Table 3.1 Oxidative Stress Western Blot Results Comparison

	Wild Type	Triple Mutant	N296N	Δ280K	P301L
P199/200	↑	↑	↑	↑	↑
GSK3β	↑ (time independent)	↑ (time independent)	↑	↑	↑ (time independent)
GSK3β-ser9	↓	↓ (time independent)	No change	↓	↓
HSP70	↑ (time independent)	No change	No change	↑	↑ (time independent)

Chapter 3

CONCLUSION AND FUTURE DIRECTIONS

3.1 Conclusion

This *in vivo* work focused on the role of a cellular response pathway, the oxidative stress pathway, in hyperphosphorylation of tau, a hallmark of neurodegenerative diseases. While oxidative stress has been observed in Alzheimer's disease [30], a mechanistic role for it in disease pathogenesis has not been demonstrated. The work in this thesis attempted to establish a link between oxidative stress and tau.

These results establish a connection between oxidative stress and tau phosphorylation, and demonstrate that different pathways are affected by oxidative stress. Therefore we can hypothesize that oxidative stress may play a causative role in tau hyperphosphorylation. Now that background work has been completed more intensive work can begin on these pathways.

3.2 Future Work

Continuing analysis in comparing the wild-type results to the different variants is necessary to verify if tau variants interact with cellular quality control differently than wild-type tau. This will allow determination as to whether oxidative stress and tau mutations act synergistically to increase the severity of tau dysfunction.

Future work needs to be performed in order to determine the pathway and causality of oxidative stress and tau phosphorylation. From the results and Figure 1.2, we can see that all three pathways may be involved in the effects of oxidative stress on tau pathogenesis, as tau chaperones and kinases were all effected. To obtain more detailed pathway information, upregulation of the oxidative stress response pathways can be measured

by qPCR studies of HO-1 mRNA. When combined, this data will enable us to examine the activation of refolding or degradation pathways following oxidative stress, and relate these changes to tau homeostasis.

Also, reporter gene studies could be performed in order to study the real time effects of oxidative stress and how the different pathways are activated in response to oxidative stress. One possibility is the Cignal Pathway Reporter kit (SABiosciences), which provides an ARE-GFP vector. With this vector transfected into our TREx-293 tau cells, we could monitor oxidative stress in real time along with the changes in tau phosphorylation, tau kinases, and tau chaperones.

Once we have established which pathways are affected by tau mutations, testing whether the same pathways or steps are altered in transgenic mice can begin. Changes in protein and mRNA levels in brain tissue from P301S and R1.40 (APP+K670N/M671L) transgenic mice, relative to wild-type mice can be monitored. The P301S mice show a neurodegenerative phenotype with limb weakness and brain atrophy after 3 months of age progressing to paralysis at 7-10 months of age, coincident with tau filament formation [15]. The R1.40 mouse shows amyloid deposition by 14 months, primarily in the cortex [31].

Long-term goals of this project are to define cellular pathways of tau homoeostasis, focusing on degradation pathways, tangle formation, and oxidative stress response, and to identify the steps in the tau pathways that are targets for therapeutic intervention. It is evident that the phosphorylation of tau plays a key role in disease pathogenesis; however, the initiation and progression of tauopathies is not clear. We will investigate how oxidative stress and mutations play a role in tau phosphorylation and function. More work is clearly needed to fully elucidate how tau processes are perturbed and contribute to neurodegeneration.

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