EFFECTS OF ESTRADIOL ON TAU UPTAKE IN CELL CULTURE

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Abstract

Tau is a microtubule associated protein that plays an important role in regulating the dynamic instability of the cytoskeleton. In adult neurons, tau is found most abundantly in axons, where it helps to organize microtubule assembly and stabilization. Post-translational modifications of tau, mainly phosphorylation, have been shown to disrupt such stabilization and regulation, and hyperphosphorylation of tau can lead to pathological results. Once phosphorylated, aggregated tau can propagate between cells, inducing tau hyperphosphorylation in a prion-like manner. Hyperphosphorylation and aggregation of tau have been linked to neurodegenerative disorders (characterized as tauopathies) such as Alzheimer’s Disease.

Nearly two-thirds of all those diagnosed with Alzheimer's Disease are women. Recent research proposes the existence of sex differences in brain-wide accumulation of tau, likely due to an accelerated spread. Moreover, a large body of evidence suggests that estradiol has a neuroprotective role in the central nervous system, including the hippocampus, and estradiol levels are lower in post-menopausal women. Taken together, we hypothesized that estradiol may serve a neuroprotective effect that impacts tau uptake and is reduced following menopause. We used cell culture to investigate whether pre-incubation with 10nM estradiol at the 30 minute and 24-hour time periods had an effect on the speed tau uptake for 0N4R wildtype and phosphorylated tau. We found that while there was no significant difference in speed of uptake between the estradiol-treated and control well for the 30-minute treatment period, there was a significant difference for the 24-hour treatment period following both 0N4R wildtype and phosphorylated tau administration.
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Introduction

Tauopathy Background

Tau is a microtubule-associated protein that plays a role in assembling microtubules and stabilizing the cytoskeleton of the cell. When tau undergoes post-translational modifications such as hyperphosphorylation, there are several consequences, some of which include dissociation from microtubules and formation of aggregates. These events are associated with neurodegeneration and the progression of a class of diseases characterized as tauopathies. Tauopathies are a major class of neurodegenerative diseases characterized by abnormal intracellular accumulations of the microtubule-associated protein tau.

Tauopathies include Alzheimer's Disease, tangle-only dementia, Pick Disease, Argyrophilic Grain Disease, Chronic Encephalopathy, Progressive Supranuclear Palsy, and Corticobasal Degeneration (Falcon, 2015). Of these diseases, Alzheimer's Disease is classified as the most common tauopathy.

It is estimated that ten percent of Americans age 65 or older are living with Alzheimer's Disease (Alzheimer's Association 2018). This indicates that there are currently 5.7 million people suffering from the disease, and this number is expected to increase to 14 million over the next 20 years (Alzheimer's Disease Fact Sheet). Alzheimer's disease is an irreversible, progressive brain disorder that accounts for 60 to 80 percent of dementia cases, which typically involves noticeable memory, thinking and behavioral symptoms that interfere with ability to function in daily life (Alzheimer's Association 2018). While a small percentage of cases are due to mutations in the MAPT gene (which encodes the protein tau) following a dominant inheritance pattern, the vast majority are sporadic (Goedert 2017).
The two main neuropathic lesions commonly found in patients with Alzheimer's Disease are amyloid beta plaques and neurofibrillary tau tangles. Both of these neuropathic lesions are associated with the neurodegeneration underlying Alzheimer's disease progression. Considering that Alzheimer's Disease is characterized as a tauopathy, it may seem surprising that Alzheimer's research has not always been geared towards tau-targeted therapies. Since the mid 1980s, disease-modifying Alzheimer's research was focused on reducing levels of amyloid beta plaques, which are often present extracellularly in the brains of AD patients (Giacobini 2013). While some compounds have been developed to successfully reduce amyloid-beta plaques, no intervention has produced clinically-meaningful results.

Due to the lack of success of Amyloid-Beta targeted therapies, recent research has shifted focus to tau-targeted therapies (Gong 2018). Tau and hyperphosphorylated tau are more closely correlated with cognitive performance than amyloid beta plaques, as immunization against amyloid beta plaques in adults with mild-to-moderately severe Alzheimer's Disease reduced plaques but did not improve cognitive function, while tau immunotherapy that reduces tau pathology has been shown to improve cognitive deficits in animal models of AD (Giacobini 2017). The amyloid beta model is still useful, although it is unproven and in the process of revision to incorporate the crucial role of tau as a therapeutic target.

Currently, there are no successful therapeutic interventions for AD. The only FDA approved drugs, Cholinesterase inhibitors and NMDA receptor antagonists, modestly improve quality of life throughout disease progression but do not address the underlying cause of the disease (Alzheimer's Association 2018). Continued research is
required to determine and properly address the biological mechanisms of disease progression.

**Tau**

Tau is an unusually hydrophilic, natively soluble protein containing many serine and threonine residues, which are important in phosphorylation. While it is unknown how exactly phosphorylation occurs and may lead to aggregation, it is known that tau is often phosphorylated at these serine and threonine residues (Gong 2008).

Tau is intrinsically unfolded and is encoded by the *MAPT* gene, which is located on chromosome 17 (Wang 2016). This gene undergoes mRNA alternative splicing to generate six isoforms expressed in the human brain. These isoforms differ by the presence or absence of inserts of 29 or 58 amino acids in the amino-terminal half, and the inclusion of the 31 amino acid repeat encoded by exon 10 of *MAPT* in the carboxy-terminal half (Goedert 2017). As depicted in Figure 1, this generates isoforms consisting of (0N, 1N, or 2N) referring to the N-terminus and (3R or 4R) referring to the C-terminus.

![Diagram](image-url)

**Figure 1**- The Six Isoforms of tau: The number of N-terminal projections is determined by alternative splicing of exons 2 and 3, while the number of C-terminal repeats is determined by alternative splicing of exon 10.
In terms of disease pathology, it is especially important to consider the C-terminal repeats, as they make up the microtubule-binding domain. As the name indicates, this region is specific for microtubule binding and is affected by hyperphosphorylation. The isoforms also have important implications in disease pathology, as they differ in their propensity for aggregation. This difference is based on the C-terminal repeats, as 4R tau has the greatest likelihood to aggregate and is most associated with disease pathology (Wang 2016). Over the course of some dementias, including Alzheimer's Disease and Frontotemporal Dementia, the relative proportions of three-repeat and four-repeat isoforms change significantly, where the four-repeat isoform eventually outnumbers the three-repeat isoform two to one (Ginsberg, 2006). Though most of these isoforms occur in the adult brain, the 0N 3R isoform is only found in fetal development, and 0N 4R is most common in the adult brain.

**Figure 2:** Diagram representing localization of phosphorylation and endogenous tau from axons to cell body as Alzheimer's Disease progresses.

Tau is found most abundantly in neurons, and in lower levels in glia and oligodendrocytes. The subcellular location of tau is developmentally regulated. As
illustrated in Figure 2, tau is distributed between cell bodies and neurites during development, but as the neuron matures and polarizes it is more localized in axons where it plays a regulatory role in stabilizing microtubules. As illustrated in Figure 3, it is worth noting that tau has a variety of other functions aside from helping to maintain dynamic instability (Wang 2016). In axons, tau may also play a regulatory role in axonal transport by competing with motor proteins kinesin and dynein for microtubule binding. Some tau is also found in the nucleus, where it may play a protective role in maintaining the genomic integrity of DNA. Tau is not typically found in the extracellular space or in presynaptic terminals.

**Figure 3:** Diagram representing tau's various functions in several subcellular compartments as well as disruption in these compartments with pathological tau.

Due to tau's variety of functions within the cell, phosphorylation may have several detrimental consequences. As mentioned previously, hyperphosphorylated tau has the tendency to detach from microtubules, resulting in microtubule disassembly. Moreover, the detached tau may mislocalize to presynaptic terminals, inducing synaptic dysfunction.
and possibly a reduction in the number of presynaptic vesicles and synapse loss. Similarly, phosphorylated tau may enter postsynaptic terminals and induce synapse loss here as well. Aggregated tau cannot enter the nucleus, and therefore cannot serve its protective function when phosphorylated. While these effects are a result of hyperphosphorylated tau missorting from axons, there are other mechanisms by which modified tau may induce pathology. Additionally, phosphorylation may alter tau's degradation and truncation leading to abnormally high intracellular concentrations and may also change the association of tau with its interacting partners. Hyperphosphorylated tau can bind to normal tau, enabling tau-tau interactions and forming paired helical filaments.

Research that I performed last summer provided trending evidence that rats injected with mutant tau expressed higher GFAP levels in the brain than rats injected with wildtype tau. Considering that GFAP (Glial Fibrillary Acidic Protein) levels are indicative of an immune response and that this "mutant tau" was more prone to hyperphosphorylation and aggregation, our results (as depicted in Figure 4) suggested that hyperphosphorylated tau is associated with an immune response and may have pathological implications.
It is unclear whether hyperphosphorylation precedes or is even necessary for aggregation, but both are correlated with pathological results (Narasimhan 2017). Earlier studies have suggested that tau becomes hyperphosphorylated prior to the appearance of neurofibrillary tangles, but research in recent decades has been unable to confirm this (Gong 2008). The function of each of the phosphorylation sites is yet to be elucidated, therefore there is insufficient information to make claims regarding the mechanism of phosphorylation or its role in aggregation. Regardless, it is well-established that loss of tau function is typically ascribed to post-translational modifications such as phosphorylation. Importantly, aggregates are not only associated with intracellular dysfunction, but can also enter the extracellular space and propagate between cells.

The ability of pathological tau to propagate between cells is significant in spreading of disease pathology (Holmes 2014). Tau is said to have "prion-like properties," meaning once phosphorylated tau leaves the cell of origin, it can contact a connected cell and induce further aggregation via a templated conformational change.
mechanism, alternating the conformation of tau in each neuron that internalizes it (Goedert 2017). Recent research has demonstrated that in vitro, protein aggregates are mobile and can spread between cells and induce fibrillization (Mudher 2017). One specific study showed that insoluble tau from transgenic mice induces the aggregation of soluble human tau in HEK293T cells (Falcon 2015). There is also evidence that this occurs in vivo, as another study provided evidence that tau propagates aggregate strains in cells and mice (Holmes 2014). These studies strongly suggest that tau crosses synapses, which may lead to the understanding of the role of neural networks in neurodegenerative diseases.

The mechanism of tau secretion has been the focus of several recent studies and is currently debated. The controversy lies in whether tau is released in the free soluble form or is packaged into vesicles such as exosomes (Goedert 2017). Exosomes are vesicles released into the extracellular space following fusion with the plasma membrane intracellularly, and are often implicated in intercellular communication. An early study focusing on tau secretion in HEK cells overexpressing tau suggested that tau release into microvesicles is an actively regulated process (Simon 2012), while another study examining cell culture media from HEK cells expressing human tau found soluble tau in the extracellular space but no detectable tau in exosome fraction (Chai 2012). More recently, a 2015 study demonstrated that inhibition of exosome synthesis halts tau propagation, suggesting that tau is released in exosomes (Asai 2015). In humans, a study investigating tau spread found that both uncoated tau (likely arising from neuron death) and vesicle secreted tau (consistent with a proposed exocytosis-endocytosis mechanism for tau spread) were present in the cerebrospinal fluid of patients with Alzheimer's
disease (Muñoz-Mayorga 2018). Preliminary studies in our lab showed that pre-incubation of SH-SY5Y and HEK cells at 4 degrees Celsius fully block the uptake of µM tau-AlexaFluor-488 as measured by live-cell microscopy and flow cytometry, indicating that internalization is likely due to endocytosis (Oseid 2016).

Studies have also indicated that internalization of tau may differ based on the phosphorylation state of tau. As hyperphosphorylated tau has a tendency for detachment from microtubules and aggregation, oligomeric tau may differ from monomeric tau in its propagation. A 2018 study demonstrated that the translocation of full-length tau to the extracellular side is enhanced by hyperphosphorylation (Katsinelos 2018). Enhanced secretion and internalization of phosphorylated tau may be due to association with the cell membrane leading to internalization. For our purposes, transmission of monomeric wild type and phosphorylated tau will be examined. Understanding intercellular transmission of tau may have crucial implications in sex-specific tau spread, which will be discussed later.

Role of Estradiol

Elucidating the fundamentals of tau modification and spread is crucial in developing therapeutic interventions and especially important in addressing sex differences in Alzheimer's Disease. The prevalence of Alzheimer's Disease is significantly higher in women compared to men, as recent estimates suggest that almost two-thirds of the individuals diagnosed are women (Mielke 2014). A historically common explanation for this phenomenon is that women tend to live longer and it is more common for men to die from competing causes of death earlier in life, thus only the more resilient men live past 65, which is when an Alzheimer's diagnosis is most common
(Vest 2013). This explanation fails to address important sex differences, and a considerable amount of research supports that the higher prevalence in women is affected by the interplay between age and sex (Vest 2013).

It is well-studied that estrogen may serve a neuroprotective role, although the precise mechanisms remain elusive (Alvarez-de-la-Rosa 2005). It is possible that the mechanisms involve signaling pathways that affect neurotrophic factors and synaptic plasticity or indirect effects on astrocytes and microglia (Brann 2007). This is studied not only in neurodegenerative diseases but also in general CNS injury (Raghava 2017). In fact, some of the first indirect evidence that estrogen may be neuroprotective arose from animal studies on sex differences in brain injury, one of which revealed that female gerbils showed lower incidence and less severe brain damage following carotid artery occlusion than male gerbils (Hall 1991). Studies following this confirmed these findings, as female rats and mice have shown a smaller infarct volume as compared to male rats following middle cerebral artery occlusion, and female rats have reported greater survival rates compared to males following diffuse traumatic brain injury (Alkayed 1998).

Interestingly enough, the hippocampus may be the primary site of action of estrogen on cognition. This is significant, considering that the hippocampus is one of the main brain structures of interest in studying AD due to its involvement in memory and cognition. Studies supporting this have shown increased neuronal excitability after estradiol administration to ovariectomized rats and a positive correlation of hippocampal CA1 dendritic spine and synapse density and estrogen levels following exogenous estrogen administration to ovariectomized rats and monkeys (Gould 1990). More specifically, one study found that estradiol regulates hippocampus dendritic spine density
via an NMDA receptor-dependent mechanism (Woolley 1994). On a cellular level, these results were confirmed via several approaches in each of these studies including Golgi analysis, light microscopic or electron microscopic analysis or synapses or synaptic proteins, dye-filling techniques and radio immunochemistry. A hypothesized model for the synaptic changes associated with estrogen that may underlie learning and memory is that estrogen enhances long term potentiation (which is associated with synapse density). These results are reproducible in both animal models and cell culture, as estrogen has also been shown to increase spine density and neurite outgrowth in vitro in neuronal hippocampal cell cultures (Li 2004).

Considering these data, it may seem that women should have a lower susceptibility for Alzheimer's Disease considering that they tend to have higher levels of estrogen than men. However, most women are diagnosed with Alzheimer's Disease post-menopause, after estrogen levels decline due to follicular depletion. Since menopause alters circulating levels of estrogen, the neuroprotective effects may be lost in aging women, therefore increasing their susceptibility for neurodegenerative diseases such as Alzheimer's Disease (Intiaz 2017). Estradiol plays an essential role in the neurobiology of aging, since endocrine and neural senescence tend to overlap timewise and are integrated in complex feedback loops.

Studies using transgenic mouse models of AD in which sex differences were documented suggested that aged females were more vulnerable to neuropathology than males (Mielke 2014). For instance, one study demonstrated that female 3xTg-AD mice exhibited significantly greater amyloid beta burden and larger behavioral deficits than male mice of the same age (Carroll 2010). These findings further suggest that depletion
of estrogens in women increase the vulnerability to AD pathogenesis and that estrogens exert neuroprotective actions, including protection from neuron death and promotion of select aspects of neural plasticity, some in a sex-specific manner. These findings are supported by the fact that a study from 1999 showed that estrogen replacement therapy may forestall the onset of degenerative changes associated with AD (Waring 1999).

However, a more recent study done in 2017 has failed to provide strong evidence for a protective association between postmenopausal hormone therapy and AD or dementia (Bushra 2017). It is worth noting that although a strong protective association was not established, a reduced risk of AD among subjects with long-term self-reported hormone therapy was observed.

Although some progress has been made in determining the effects of estrogen levels on neurodegenerative diseases, the mechanism as it relates to pathology is not well understood. A recent study done at the University of Vanderbilt suggested that postmenopausal women show a larger brain-wide accumulation of tau compared with men, and that this may be due to an accelerated brain-wide spread of tau (Herbers 2012). This relates to the accumulating evidence that tau has prion-like properties, enabling pathogenic tau spread throughout neural networks. This study used graph theory analysis and data from Positron Emission Tomography scans of both healthy individuals and patients with mild cognitive impairment to construct in vivo networks that modeled tau spread. The graphing analysis was used to reconstruct networks in the brain modeling tau spread, and their findings suggested that accelerated tau spread in women may demonstrate a need for sex-specific approaches for the prevention of Alzheimer's Disease. While this study is yet to be peer-reviewed, the results were presented at the
Alzheimer's Association International Conference in July. This study presents a possible explanation for the higher prevalence of AD in women that addresses tau uptake, and further research is needed to fully understand the biological mechanisms influencing these differences in tau spread.

In our experiments with cell culture, we will be focusing on estradiol (E2 or 17β-estradiol) because its decline is most prevalent in the menopausal transition and has been associated with a number of changes in the brain (Russell 2019). Estradiol is one of the three most common estrogens found in the body and is the most heavily associated with neuroprotection in animal models. Both in vivo and in vitro studies have demonstrated that estradiol can mediate tau dephosphorylation (Russell 2019). One of these studies, which was done using the cell line SH-SY5Y (human neuroblastoma cells) and primary cultures of newborn male or female rat cortical neurons, found that estradiol induced dephosphorylation at the proline-directed site of the molecule and attenuated the hyperphosphorylation of tau (Alvarez-de-la-Rosa 2005). Furthermore, an in vitro investigating alpha secretase activity in the cleavage of the amyloid precursor protein demonstrated that estradiol stimulation may result in in amyloid being processed preferentially in the soluble (and therefore less pathogenic) form 17β-estradiol is routinely used in cell culture studies (Goodenough 2013).

Previous Cell Culture Work

Previous work by Dr. Anne Robinson's lab demonstrated that several cell lines can take up recombinant tau in both the non-phosphorylated monomeric and phosphorylated state within minutes. Tau uptake was apparent by vesicle formation, as shown in the figure on the following page.
Figure 5 (left): Time before appearance of internalized tau. Cell types as indicated; Individual points (right): 1uM Alexa-488 labeled tau was added to the media cells. Punctuate structures observed within SH-SY5Y cells via confocal microscopy as early as 4-6 minutes following addition of WT monomer. correspond to different cells quantified, n>20.

It is important to note that cells were assayed for their ability to endocytose equimolar amounts of labeled bovine serum albumin (BSA-AlexaFluor-488) or free alexaFluor-488 in solution (Oseid, 2019). There was no uptake of BSA-AlexaFluor-488 or free alexaFluor-488 observed in any cell type, indicating that uptake in our experiments is tau-specific.

We will continue with this technique to observe the effects of preincubation with estradiol for several different time periods on the speed of tau uptake. While accurately mimicking pre and post-menopausal states would require other hormones such as progesterone and growth factors, we will begin with examining just the effects of estradiol. The present study aims to investigate whether the estradiol has an effect on tau uptake in vitro. Our findings may shed light on the hypothesis that differences between sexes in tau spread are influenced by hormone levels.
Methods

Experiment 1

Cell Culture

Human Embryonic Kidney Cells were cultured according to optimized media conditions described by the ATCC. Cells were cultured at 37 degrees at 5% CO2. HEK cells were pulled out of liquid nitrogen, thawed, resuspended in media and placed into a T25 flask. Cell viability was monitored over several days prior to seeding for confocal imaging. 500ul of HEK cells in media were added to several 1000ul confocal wells. Following seeding, a solution of ethanol-dissolved estradiol was prepared to be added to one confocal well for a final concentration of 10nM. The well was then placed in an incubator for varying time periods (30 minutes or 24 hours) depending on the experiment. The final 10nM final concentration of estradiol in the well is typically used to mimic its physiological concentration. A control solution was prepared with 70% ethanol. This solution was added to a different confocal well and placed in an incubator for the same period of time as the estradiol-treated well.

Imaging

Following incubation, the confocal well was placed in a live-cell imaging chamber to be imaged on a Nikon A1 microscope. In the imaging chamber, a 2uM fluorescently-labeled tau solution was added to the well to be imaged at a final concentration of 1uM. The fluorescently-labeled tau isomers varied by the experiment (0N4R wt. tau or 0N4R ptau). It is important to note that all the tau used in these experiments are monomeric and in the non-aggregated state. Fluorescently-labeled tau variants were imaged at an excitation wavelength of 488nm and an emission wavelength
of 525nm. After the tau solution was added, the live cell imaging began and recorded for at least 25 minutes at a magnification of 60x.

**Analysis**

The imaging session was analyzed using NIS Elements. Tau uptake was measured by determining the time that the first vesicle appeared in a cell. The time of the first vesicle entry was recorded for five to ten cells. Prism graph pad was used to record and analyze the speed of vesicle entry. The speed of tau uptake was compared between the estradiol treated well and the ethanol treated well.

![Figure 6: Left: HEK-293 cells at the beginning of the imaging session, seconds after tau was added to the well. Right: HEK-293 cells at the end of the imaging session, once tau is present in vesicles in the cell.](image)

**Experiment 2**

**Cell Culture**

To determine the validity of the control well, the effects of ethanol on tau uptake were measured. In this experiment, an ethanol-treated well was compared with a completely untreated well. Cells were obtained and cultured as described in experiment 1. One well was treated with 70% ethanol, while another well contained only 250ul HEK
media. The ethanol-treated well had an incubation period of 30 minutes. The confocal well was placed in a live cell imaging chamber following incubation.

**Imaging**

In the imaging chamber, 250ul of a prepared fluorescently-labeled tau solution containing phospho tau was added to each well for a final concentration of 1uM. Fluorescently-labeled tau variants were imaged at an excitation wavelength of 488nm and an emission wavelength of 525nm. After the tau solution was added, the live cell imaging began and recorded for 30 minutes at a magnification of 60x.

**Analysis**

As done in experiment 1, tau uptake was measured by determining the time that the first vesicle appeared in a cell. The time of the first vesicle entry was recorded for six cells. NIS Elements was used for imaging analysis and Prism graph pad was used to record and analyze the speed of vesicle entry. The speed of tau uptake was compared between the ethanol treated well and the untreated well.

**Experiment 3**

**Cell Culture**

Following the results of Experiment 2, Experiment 1 was adjusted to starve cells of steroid components in media and to eliminate the effects of ethanol on tau. The latter was accomplished by using water-soluble estradiol in place of ethanol-dissolved estradiol. Estradiol is rendered water soluble by encapsulation in a cyclodextrin ring, with 0.45mg estradiol per gram solid. When this complex is dissolved in water, estradiol is released from the complex into solution. Therefore, the molarity of the estradiol component was used to calculate the amount needed to achieve a final molarity of 10nM.
in the confocal well. Cells were obtained and cultured as described in experiments 1 and 2. Additionally, new media was made to starve the cells of steroids for three days prior to imaging. Culturing in the new media, which contained 10% charcoal-stripped FBS and 90% phenol red-free DMEM, curtailed potential estrogen-like actions of media components. The new media lacked growth factors such as L-Glutamine, an amino acid that is an important energy source for cells in culture. A water-soluble estradiol and charcoal-stripped media solution was prepared for a final concentration of 10nM once added to the well. This solution was then added to one confocal well, which was placed in an incubator for varying time periods (30 minutes or 24 hours) depending on the experiment. The control well was untreated.

**Imaging**

Following incubation, the confocal well was placed in a live-cell imaging chamber to be imaged on a Nikon A1 microscope. The imaging procedure for this experiment was the same procedure as described in previous experiments.

**Analysis**

The imaging session was analyzed in the same way as described in previous experiments.

**Results**

The speed of tau uptake by HEK cells treated with estradiol, treated with ethanol and untreated was determined using video from the live imaging session. NIS elements was used to manipulate the speed of the 25-minute video and the zoom in on one cell at a time. The video was analyzed at several time points until the time of the first vesicle present could be determined. Between 5 and 10 cells were analyzed per well. Prism was
used to visualize results from the experiment and determine whether there were any significant differences. This technique was used to analyze all experiments.

Experiment 1: Tau Uptake in Ethanol-Dissolved Estradiol-Treated Well vs. Ethanol-Treated Well

An unpaired t-test with Welch's correction revealed a significant difference between the estradiol-treated well for the 30 minute but not the 24-hour time period. The cells in this experiment were cultured in media containing phenol red and FBS that was not charcoal-stripped. As shown in figure 7.1, results from the 30-minute experiment demonstrated trending evidence for faster tau uptake in the estradiol-treated well. For this experiment, p = <0.0001.

Figure 7.1: Tau (0N4R wildtype) Uptake following 30-minute Incubation Period

Time until first vesicle present for estradiol-treated well and ethanol-treated control well. N=6 for both the estradiol and control well.

As shown in figure 7.2 on the following page, there was little difference between the estradiol-treated well and the ethanol-treated well following a 24-hour incubation period. For this experiment, p = 0.4171, which is far from significant.
Experiment 2: Tau Uptake in Ethanol-Treated Well vs. Untreated Well

An unpaired t-test with Welch's correction revealed a significant difference between the ethanol-treated well and the untreated well. This experiment was run to assess the effects of ethanol on rate of tau uptake and determine the validity of our control. As shown in figure 8.1, \( p = 0.0146 \) for this experiment. Figure 8.2 illustrates the unhealthy appearance of the ethanol-treated HEK cells as compared with the untreated HEK cells, which exhibit healthy morphology. Further experiments were adjusted such that water-soluble estradiol was used in comparison with an untreated well and no cells were treated with ethanol.

Figure 7.2: Tau (0N4R wildtype) Uptake following 24-hour Incubation Period

Time until first vesicle present for estradiol-treated well and ethanol-treated control well. \( N=5 \) for both the estradiol and control well.

Figure 8.1 (left): Tau (0N4R wildtype) Uptake following 30-minute Incubation Period: Time until first vesicle present for ethanol-treated well and untreated control well.
Experiment 3: Tau Uptake in Water-Soluble Estradiol-Treated Well vs. Untreated Well

For the experiments involving wildtype tau, an unpaired t-test with Welch's correction did not reveal a significant difference between the estradiol-treated well and the untreated well for the 30-minute time period. This experiment was adjusted both to remove the ethanol component of the experiment and estrogenic components of the media. Charcoal-stripped FBS serum and DMEM without phenol red were used in these experiments. As shown in figure 9.1 for the 30 minute time period, there was not a significant difference for the control vs. estradiol treated well, as p= 0.4917.

Figure 8.2 (right): Cells were imaged at 60x using a Nikon A1 confocal microscope. Left image shows ethanol-treated HEK cells, while right image shows untreated HEK cells with normal morphology.

Figure 9.1: Tau (0N4R wildtype) Uptake following 30-minute Incubation Period
Time until first vesicle present following incubation in water-soluble estradiol after three days in charcoal-stripped, phenol red-free media.
As illustrated in figure 9.2, the p value for the unpaired t test with Welch's correction was p= 0.0299, indicating a significant difference.

**Figure 9.2: Tau (0N4R wildtype) Uptake following 24-hour Incubation Period**
Time until first vesicle present following incubation in water-soluble estradiol after three days in charcoal-stripped, phenol red-free media.
N=6

We also examined uptake of 0N4R phosphorylated tau following a 24-hour incubation period. For this experiment, there was a significant difference between the control and the estradiol-treated well, as p= 0.0214.

**Figure 9.3: Tau (0N4R Ptau) Uptake following 24-hour Incubation Period**
Time until first vesicle present following incubation in water-soluble estradiol after three days in charcoal-stripped, phenol red-free media.
N=6
For this set of experiments, we not only looked at time until first vesicle present, but also at the total number of vesicles present per cell at the 15-minute mark of the imaging period, when most tau vesicles had been taken up by the cells. In this analysis, there was not a significant difference between the estradiol-treated and untreated wells for either the wildtype or the phosphorylated tau.

**Figure 9.4 (Left) and 9.5 (Right):** 0N4R Tau (wildtype Left, Phosphorylated Right) vesicle present at 15 min interval of imaging session.

**Discussion**

These experiments were performed to investigate the possible effect of estradiol preincubation on tau uptake in HEK cells based on preliminary research done by Danny Oseid in Dr. Robinson's lab, which demonstrated that monomeric wildtype and phosphorylated tau could be taken up by several cell lines within minutes (Oseid 2016). Based on recent research done at Vanderbilt University suggesting that there are sex differences in speed of tau spread, we hypothesized that physiologic concentrations of estradiol may have an effect on tau uptake (Herbers 2019). In our first experiments, we cultured HEK cells in media containing phenol red that had not been charcoal-stripped then incubated the cells in ethanol-dissolved estradiol prior to imaging. Previous studies
commonly used estradiol concentrations between 1 and 10nM, so we used a final concentration of 10nM in each well receiving the treatment.

This first experiment revealed a significant difference with the estradiol well showing quicker tau uptake, however we were skeptical of whether this effect was due to the estradiol alone or if the ethanol had any effect on cell viability. If so, our data would not be representative of healthy HEK cell activity. While it is well-known that ethanol is a threat to the health of cells, our concentration was so low we did not expect it to have an effect. However, we noticed abnormal morphology in the HEK cells containing ethanol and compared with completely untreated cells, leading us to run a control experiment measuring tau uptake in a completely untreated well versus the control concentration of ethanol. When this experiment revealed a significant difference with the ethanol well demonstrating slower tau uptake, we ordered water-soluble estradiol to use for all future experiments. Further, we switched to media containing charcoal-stripped FBS and phenol-free DMEM for future experiments, as this reduces variability in the estrogenic components of media. When this experiment was performed again with these adjustments, there was no significant difference for the 30-minute incubation period.

Considering that many media components exert estrogen-like actions, cell-culture experiments are susceptible to the bias of their hormonal environment if these effects are not accounted for. Specifically, concentrations of estrogens and phytoestrogens differ due to FBS serum, which has variable composition. To minimize this effect, FBS serum can be charcoal filtered. While charcoal-filtering aids in avoiding the impact of hormones, there is still some variability in hormone concentrations depending on the amount of charcoal used and filtering time (Sikora et al., 2016). Another detrimental effect of
charcoal filtering is depletion of growth hormones and vitamins which are important for cell viability. Irrespective of this, charcoal filtering is still considered necessary for cell culture experiments involving hormones.

Furthermore, phenol red, a pH indicator used in most commercial media, resembles some nonsteroidal estrogens and has the tendency to bind to and activate estrogen receptors, which is problematic in experiments involving hormones. Cancer cell studies have demonstrated this effect in a dose-dependent manner, as MCF-7 cells were more proliferative in media containing phenol red versus media with less phenol red (Wesierska-Gadek et al., 2007). While using phenol red free media makes it more difficult to monitor the pH of culture, it introduces less confounding variables than media containing phenol red for steroid experiments.

A less commonly addressed confound is the use of plastics in cell culture experiments. Most plastic ware contains polystyrenes, which release phenolic components into the media and possibly influence estrogenic pathways (Soto et al., 1991). The weak estrogens released into the medium may attenuate the effects of exogenous estrogens used in experiments, and while these effects are worth noting, the use of plastics would be rather difficult to avoid.

Taking all of this into consideration, our second experiments were modified to include water-soluble estradiol, charcoal-stripped FBS and phenol-red DMEM. Cells were still stored and cultured for 48 hours in the previously used media containing all necessary vitamins and growth factors, but were starved of steroids in the new media for three days prior to imaging. These experiments investigated the difference in speed of tau uptake over the 24hr and 30min time period with monomeric nonphosphorylated and
phosphorylated tau. The importance of investigating non phosphorylated versus phosphorylated tau lies in the fact the phosphorylation of tau may promote its internalization (Oseid 2016). The results of Daniel Oseid's experiments measuring tau uptake across various cell lines suggested that phosphorylated tau may associate at higher levels to the surface of cell membranes, which could increase their endocytosis. We were interested in investigating whether this effect was maintained after preincubation with estradiol. Interestingly enough, there was a significant difference between the estradiol and untreated well for the 24-hour period following both wildtype and phospho tau administration for the unpaired t-test with Welch's correction. Additionally, there was no significant difference between the estradiol treated and untreated well for the 30-minute time period in the third experiment.

It is worth noting that all of the tau used in our experiments is monomeric rather than oligomeric. This is significant as the spreading of tau that is associated with disease progression tends to be aggregated, or oligomeric. Studies have demonstrated that it is the misfolded or aggregated tau that may act as a template and alter the conformation of tau in each neuron that internalizes it, and therefore it is the aggregated tau that may be important in spread of pathology (Goedert 2017). There is controversy surrounding whether monomeric tau spread is also detrimental to cells. While some reports have shown that the uptake of non-aggregated tau can induce morphological changes and exert neurotoxicity (Michel 2014), others have suggested that monomeric tau uptake generally is not toxic and is well-tolerated by recipient cells (Evans 2018). In one study, neurons were seeded with full-length human tau oligomers and monomers, and chronic effects on neuronal function and viability were observed over time (Usenovic 2015). Oligomer-
treated neurons showed an increase in aggregated and phosphorylated pathological tau, and these effects were associated with neurite retraction, synapse loss, aberrant calcium homeostasis, and imbalanced neurotransmitter release. The results of this study demonstrated that tau oligomers but not monomers induce accumulation of pathological, hyperphosphorylated tau. Taken together, our experiments may not be representative of the type of tau spread that is considered to be pathological in vivo.

A study examining oligomeric tau propagation in vivo and in vitro found that the initiation of tau misfolding and aggregation in vivo is complex and does not translate to straightforward formation of aggregates in vitro. While cell culture is a well-accepted method for studying mechanisms, it is worth noting that translation to in vivo is sometimes difficult. Furthermore, the cells used in our experiments were Human Embryonic Kidney cells, which do not ideally represent pathological tau spread in the brain. While it has been established in previous literature that HEK293 cells can serve as a model for tau propagation, other factors, such as the existence of synapses, may play a role in neuronal cells. Ideally, hippocampal cell culture would be used to simulate tau spread in vivo. That being said, HEK cells are generally representative of somatic cells in vivo.

Therefore, while our experiments suggest that estradiol has an effect on tau uptake for the 24-hour time period but not the 30-minute period in monomeric tau uptake in HEK cells, it is possible that estradiol may not serve the same role in tau uptake in different cell lines or in vivo.

Another factor that must be considered is the presence of hormones and growth factors besides estradiol that decline post-menopause. As previously discussed, our
experiments were not representative fully of the *in vivo* hormonal environment, but they were based on the hypothesis that the post-menopausal hormonal environment may increase women's susceptibility for Alzheimer's Disease. Therefore, it is important to note other factors at play postmenopausally. One study aiming to mimic the pre- and post-menopausal hormonal environment in human dermal fibroblasts incubated cells in media containing 17β-estradiol, progesterone, dehydroepiandrosterone, growth hormone and insulin-like growth factor-1 at concentrations corresponding to those of non-menopausal women's sera and then of menopausal women's sera (Remoue 2013). To make any suggestions regarding the role of menopause would be incomplete without considering the possible role of any of these factors.

Overall, our results cannot be properly interpreted without the consideration of monomeric rather than oligomeric tau, the cell type used, the hormones not investigated, and *in vitro* versus *in vivo* conditions. Our results do suggest that preincubation of HEK cells with a 10nM concentration of estradiol for 24-hour time period, following 3 days in charcoal-stripped, phenol red-free media, has an effect on the speed of monomeric phosphorylated and non-phosphorylated tau uptake. This result for both phosphorylated and non-phosphorylated tau administration showed a significant difference, but with different effects on the rate of uptake. Considering that there was a significant difference only in the experiments with the longer time period, we suspect that this may be due to a genomic effect of estrogen, rather than a non-genomic effect that would be attributable to membrane action. Additionally, it took longer for the first vesicle to appear in the estradiol treated wells compared with untreated wells following administration of wildtype 0N4R tau, but uptake of phosphorylated 0N4R tau was quicker in the estradiol
treated well compared with the untreated well. We believe this provides trending
evidence that preincubation with estradiol for longer time periods may have an impact on
tau uptake, and that this effect is different for phosphorylated tau and non-phosphorylated
tau.


