Spring 2018

Characterizing Tau in the Nucleus

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Characterizing Tau in the Nucleus

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Science and Mathematics
James Madison University

Shaw Grindle Camphire and Madeline Louise Henwood

May 2018

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at the Annual Neuroscience Meeting in D.C. on 11/11/17

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ABSTRACT

A hallmark of Alzheimer’s Disease (AD) is the aggregation of hyperphosphorylated tau protein into neurofibrillary tangles. Tau localizes to both the cytoplasm and the nucleus of neuronal cells; however, its nuclear role has not been fully defined. Tau has recently been shown to bind to purine-pyrimidine (R/Y) repeats in DNA and stabilize them into Z-DNA. Evidence from our lab suggests that the binding of tau to R/Y repeats causes transcriptional changes of the NOS1 gene. Six major isoforms of tau exist in neurons. These isoforms fall into two groups, denoted as 3R tau and 4R tau, and are found at an equal ratio in healthy whole-cell extracts. During AD progression, an imbalance of 3R and 4R tau occurs, along with changes in NOS1 expression and a buildup of Z-DNA in severe AD patients. Based on studies of mouse tau and our lab’s previous work, we hypothesized that the 3R and 4R tau would be found in equal ratios in the nucleus of normal human cells, and that tau would bind to the NOS1 R/Y repeat. To test these hypotheses, western blotting of nuclear proteins from human neuroblastoma cells (SK-N-MC) and antibodies for specific tau isoforms were used to examine which isoforms were found in SK-N-MC cells. Additionally, electrophoretic mobility shift assays (EMSAs) were developed to better understand tau’s ability to bind R/Y repeats using the R/Y repeat from the NOS1 gene. The western blots showed that the 3R isoform of tau predominates in SK-N-MC cells, that similar tau isoforms are present in the nuclear, cytoplasmic, and chromatin-bound fractions, and that a majority of nuclear tau exists bound to DNA. Our EMSAs showed that some nuclear protein binds the NOS1 R/Y repeat, this protein has not been identified. Future experiments will use western blotting to characterize nuclear tau in other cell lines, continue improving our EMSAs, and identify the NOS1 R/Y repeat binding protein.
INTRODUCTION

Alzheimer’s Disease (AD), an incurable neurodegenerative disorder, is the sixth leading cause of death in the United States. AD can significantly impair one’s ability to think, remember, communicate, and carry out daily activities. It is projected that by 2050, the number of Americans living with AD could rise to 14 million (Alzheimer’s Association, 2018). Due to the severity of this disease and the continuous increase in its incidence, it is critical that we investigate the molecular mechanisms of the neurodegeneration in patients with AD.

A major hallmark of AD is the aggregation of hyperphosphorylated tau protein into neurofibrillary tangles (NFTs) (Lee et al., 2001). Tau, or tubulin-associated unit, was first discovered as a protein factor that promotes the stability of microtubules in the cytoplasm, though it has been discussed to have synaptic and nuclear localizations as well (Lippens et al., 2016; Sultan et al., 2011; Vasudevaraju et al., 2012; Liu and Gotz, 2013). Tau binding to microtubules is regulated by phosphorylation. Phosphorylated tau dissociates from microtubules and allows the microtubules to depolymerize. Under pathological conditions, tau phosphorylation is increased, resulting in destabilizing microtubules and potentially leading to neuronal collapse (Guo et al., 2017). Moreover, sometimes tau can be hyperphosphorylated in disease. Hyperphosphorylated tau can be truncated and, therefore, stay in the cytoplasm, often aggregating into NFTs. It is unknown if free truncated tau or the NFTs themselves are more toxic to the cell. Some studies suggest that NFTs are toxic, while others suggest that NFTs are a protective cellular mechanism against free truncated tau (Mania et al., 2015). Regardless of the uncertainty surrounding NFTs’ effects, the presence of NFTs are central to the diagnosis of AD and are highly correlated to memory loss occurring in AD (Guillozet et al., 2003).
Human tau is encoded by the \textit{MAPT} gene located on chromosome 17. In neurons, the tau protein has six major isoforms generated by alternative splicing. A seventh isoform is also found in non-neuronal cells (Goedert \textit{et al.}, 1989; Janke \textit{et al.}, 1996). The six major isoforms vary due to the presence or absence of exons 2, 3, and 10. Exons 2 and 3 contain N terminal domains. Isoforms lacking exon 2 and 3 contain 0 N domains, while isoforms containing exon 2 contain 1 N domain, and finally isoforms containing both exon 2 and 3 have 2 N-terminal domains. Isoforms containing exon 10 have four microtubule binding domains (4R) and isoforms missing this exon have three microtubule binding domains (3R) (Figure 1).

The six major isoforms are denoted 0N4R (2-, 3-, 10+), 1N4R (2+, 3-, 10+), 2N4R (2+, 3+, 10+), 0N3R (2-, 3-, 10-), 1N3R (2+, 3-, 10-), and 2N3R (2+, 3+, 10-). The big tau isoform is the only isoform that includes exon 4A.

In healthy human adult brain tissue, the 3R and 4R isoforms exist at an equal ratio. However, it has been observed that the ratio between the 3R and 4R is distorted in the AD brain, suggesting that these isoforms have different functions (Hong \textit{et al.}, 1998).
identified as an important area of research, and a model that has been used is the brains of rodents. Unfortunately, adult rodent brains only contain the 4R isoforms of tau, making it difficult to compare studies done in rodents and those done in humans. In the nucleus of mice neurons, the 1N4R isoform is the most predominant isoform, though 0N4R and 2N4R were also found (Liu and Gotz, 2013). Therefore, furthering our understanding of the isoforms of tau that are naturally present in the nucleus will help us increase our knowledge of nuclear tau and give insight how an imbalance of subunits in AD may affect nuclear functions.

Several different potential roles for tau have been found in the nucleus. Tau’s DNA binding is known to participate in the repair process of cells. One group has found that tau binds to DNA, potentially protecting DNA from damage caused by double strand breaks (Liu and Gotz, 2013). Although the nature of this protective mechanism is unknown, Liu and Gotz showed that higher levels of tau are associated with less double-stranded DNA breaks, and conversely less tau is associated with more double-stranded DNA breaks. Other studies have suggested protective roles for tau as well. Sultan et al. demonstrated that tau binding DNA protected it against heat shock induced double strand breaks, suggesting that tau is critical to early stress responses in neuronal cells (Sultan et al., 2011). Another study proposed that tau localized in the nucleus and the plasma membrane of neurons has a role in DNA repair and inter-neuron signaling through an unknown process that could be regulated through nuclear processes (Hanger et al., 2014). The 1N tau isoform shows the most affinity for DNA, likely being the greatest protector of DNA (Liu and Gotz, 2013). Tau has also been studied as a potential therapeutic target for the treatment and prevention of other neurological pathologies like epilepsy (Sotiropoulos et al., 2017). Thus, it is possible that alteration in tau during AD may be responsible for damaging changes in DNA structure that contribute to AD.
In addition to a potential role in protection of DNA, another nuclear function of tau has been shown to involve DNA binding. It is now known that tau in its unphosphorylated form localizes to the nucleus, although a specific DNA binding site for tau has not yet been identified. Several studies suggest tau binds preferentially to purine-pyrimidine (R/Y) repeats in the DNA (Sultan et al., 2011; Vasudevaraju et al., 2012; & Liu and Gotz, 2013). R/Y repeats can often be found in promoter regions and can potentially cause DNA in the repeat region to switch into alternative forms, such as Z-DNA, changing gene transcription (Cox et al., 2012). It has been shown that the tau protein is capable of binding to and stabilizing a R/Y oligonucleotide in both the B-DNA and Z-DNA conformation. Moreover, AD neurons in late-stage patients contain excessive Z-DNA, as identified in circular dichroism studies of post-mortem brains (Vasudevaraju et al., 2012). This suggests that tau binding to DNA may change as the disease progresses.

Tau has also been suggested by our lab to regulate the transcription of the nitric oxide synthase gene (NOS1). The NOS1 enzyme produces the secondary messenger nitric oxide (NO), which is crucial for the regulation of a variety of body functions including controlling blood pressure, long-term memory, and synapse formation (Toda and Okamura, 2003; Prast, 2001). NOS1 transcription must be tightly regulated, as excess NO can have neurotoxic effects, while low levels can have neuroprotective effects (Sultana et al., 2006). NOS1 is transcribed from 12 different promoters (1A-1L), one of which, 1F, has a TG repeat polymorphism which can range in size from 40-65 repeated TG nucleotides (Hall et al., 1994). A smaller polymorphic repeat in the 1F promoter leads to an increased risk of developing AD and Parkinson’s disease, another neurodegenerative disease (Galimberti et al., 2008, Rife et al., 2009). Work previously done in our lab using promoter deletion studies show that a cell with higher expression of tau also has a
change in \textit{NOS1} transcription, though this only occurs when the R/Y repeat is present in the promoter region of \textit{NOS1} (Weaver and Deal, 2016). This has been an area of interest to our lab, as not only does \textit{NOS1} contain this type of R/Y repeat, but many of the genes in Alzheimer’s do (Kimpara \textit{et al.}, 1997; Lambert \textit{et al.}, 1998; Ueki \textit{et al.}, 2007). Thus, it is of great importance to better understand how this repeat functions and gain evidence about the mechanism by which the change in \textit{NOS1} expression occurs. Whether \textit{NOS1}’s R/Y repeat is able to interact directly with the tau protein or another protein that is affected by changes in tau is unknown. Studying tau’s potential interaction with the \textit{NOS1} could give greater insights as to a new gene regulation mechanism for tau in the nucleus during Alzheimer’s disease, beyond tau’s cytosolic role in stabilizing microtubules.

Other than the data from our lab, no other studies have been done to show that tau can regulate transcription, but several other groups have suggested this possibility. One study examined possible ways through which tau may act as a transcription factor. Sultan \textit{et al.} observed that tau binds into the minor groove of DNA, which causes a kink in the strand that helps promoters to bend and interact with transcription factors (Sultan \textit{et al.}, 2011). To more completely understand the role of tau in the nucleus and its binding mechanism to R/Y repeats, a DNA binding study is necessary.
Specific Aims:

Aim 1: To determine the isoforms of tau normally found in the nucleus of human SK-N-MC cells.

While six major isoforms of tau (4R isoforms with 0N, 1N or 2N, and 3R isoforms with 0N, 1N, or 2N domains) have been identified in whole-cell extracts of human neurons, the tau isoforms found specifically in human nuclei have not yet been identified. Although mouse studies have found a predominance of the 1N4R isoform, mice do not contain any of the 3R domains. Based on studies of mouse neurons and the known ratios of 3R to 4R in humans, we hypothesize that the 1N3R and 1N4R tau isoform will be predominate in the human nucleus (Liu and Gotz, 2013).

To test this hypothesis, SK-N-MC cells, human neuroblastoma cells, were used because they have high levels of nuclear tau and have been used in previous studies to understand tau function. Proteins from these cells were fractionated into cytoplasmic and nuclear portions and run on SDS-page gels. Western blots using antibodies specific to different tau isoforms were used to identify which isoforms are found in the nucleus.

Aim 2: To determine the possible transcription factor(s) that bind to the NOS1 polymorphism.

It is well known that tau has DNA binding affinity, especially towards sequences with R/Y repeats. From previous studies in our lab, we know that either tau or another agent that is regulated by changes in tau expression can change the expression of the NOS1 gene with a R/Y repeat polymorphism in its promoter region (Weaver and Deal, 2016). However, the mechanism by which either tau or another agent is changing the expression of this gene is unknown. The R/Y repeat of the NOS1 gene is rich in TG nucleotide repeats, which can form both B-DNA and
Z-DNA (Bothe et al., 2011). Tau has been shown to have an affinity towards both B-DNA and Z-DNA, though only high levels of Z-DNA are found in Alzheimer’s brains (Vasudevaraju et al., 2012). In studies which analyzed tau’s affinity towards R/Y repeats, it was found that tau has the greatest affinity towards TA nucleotide repeats, followed by TG, AC, and CG, in that order (Sultan et al., 2011; Vasudevaraju et al., 2012). Based on studies of tau’s DNA binding affinities and previous work in our lab, we hypothesize that it is tau binding the R/Y repeat of NOS1, acting as a transcription factor to change the gene’s expression.

To learn more of how tau or another agent could be changing NOS1 expression, electrophoretic mobility shift assays (EMSA) will be used to determine the nature of the binding particle through modifying methods outlined by Dent and Latchman, and Berman et al. (1993; 1987). The goal of each EMSA is to determine a difference in mobility between control samples of only DNA, referred to as just DNA controls (JDC), and samples containing both the DNA and potential binding proteins, referred to as mobility shift attempts (MSA). Nuclear fractions from SK-N-MC cells will be run on an agarose gel with a short piece of DNA containing the NOS1 polymorphism (NOS1 probe) to act as MSA samples and compared to JDC samples containing only the NOS1 probe in order to determine a band shift, providing evidence that something is binding to the NOS1 polymorphism. Furthermore, a secondary band shift utilizing a total tau antibody, or a purified sample of tau, will be used to show whether tau is or is not the binding particle for the NOS1 probe.
METHODS

SK-N-MC Cell Maintenance:

SK-N-MC (human neuroblastoma) cells were grown in 8mL of Eagles Modified Minimal Medium with 2mM L-glutamine, 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin on a 100 mm tissue-culture treated cell culture dish and harvested with 0.25% trypsin-EDTA. For maintenance, media was vacuumed off the cell plates at 70-90% confluency and replaced with 2mL of 0.25% trypsin-EDTA. After roughly 60 seconds of trypsinization, the trypsin was removed and 4mL of media was added to rinse the cells from the plate. These cells were split into two or more plates and media was added to make a total volume of 8mL.

Cellular Fractionation and Total Protein Assay:

Two separate kits were used to obtain cellular fractions. The Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Kit for a collection of two fractions containing the cytoplasm and the nucleus; and Thermo Scientific Subcellular Proteion Fractionation Kit for Cultured Cells was used to collect five subcellular fractions containing the cytoplasm, membrane, nucleus, chromatin, and cytoskeleton. In the procedure for both kits, extra care was taken to never disturb the pellet while collecting supernatants, and to leave the pellet completely absent of supernatant after collection. Immediately following each step which required a centrifugation and subsequent collection of the supernatant, a second, short centrifugation was added in order to ensure that all liquid was removed from the pellet. During the second removal of supernatant from the pellet, there were cases in which the pellet had to be disturbed in order to leave the pellet completely dry. When the pellet must be disturbed, the supernatant collected was
disposed of as to not contaminate any fractions. After the completion of all steps in either fractionation protocol, each fraction was immediately analyzed with a total protein assay.

**Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Kit (2FK):**

All solutions were ice-cold, all samples were kept on ice, all incubations were at 4\(^0\) C, all vortexing was done on the highest setting, and Thermo Scientific Halt™ Protease Inhibitor Cocktail was added to all solutions unless otherwise noted. Cytoplasmic extraction reagent I (CERI), cytoplasmic extraction reagent II (CERII), and nuclear extraction reagent (NER), were all used. Each buffer added was adjusted to the packed cell volume in the sample.

Roughly 1-10x10\(^6\) cells suspended in PBS (no protease inhibitor) were centrifuged at 500 x g for 2-3 minutes. The supernatant was discarded and CERI was added. The sample was then vortexed for 15 seconds to resuspend pellet and incubated for 10 minutes. CERII (no protease inhibitor) was added to the sample, vortexed for 5 seconds, incubated for 1 minute, vortexed for another 5 seconds. The sample was centrifuged at 16,000 x g for 5 minutes and the supernatant (cytoplasmic extract) was collected. NER was added to the pellet and resuspended through a process of 4 consecutive 15 second vortexes and 10 minute incubations for a total 40 minutes. The sample was then centrifuged at 16,000 x g for 10 minutes and the supernatant was collected.

**Thermo Scientific™ Subcellular Protein Fractionation Kit for Cultured Cells (5FK):**

All solutions were ice-cold, all samples were kept on ice, all incubations were at 4\(^0\) C and done with gentle mixing, all vortexing was done on the highest setting, and Thermo Scientific Halt™ Protease Inhibitor Cocktail was added to all solutions unless otherwise noted. Cytoplasmic extraction buffer (CEB), membrane extraction buffer (MEB), nuclear extraction buffer (NEB), and pellet extraction buffer (PEB) were all used. Each buffer added was adjusted to the packed cell volume in the sample.
Roughly 1-10x10⁶ cells suspended in PBS (no protease inhibitor) were centrifuged at 500 x g for 2-3 minutes. The supernatant was discarded, CEB was added, and incubated for 10 minutes. The sample was centrifuged at 500 x g for 5 minutes and the supernatant (cytoplasmic extract) was collected. MEB was added to the pellet, vortexed for 5 seconds, and incubated for 10 minutes. The sample was centrifuged at 3000 x g for 5 minutes and the supernatant (membrane extract) was collected. NEB was added to the pellet, vortexed for 15 seconds, and incubated for 30 minutes. The sample was centrifuged at 5000 x g for 5 minutes and the supernatant (soluble nuclear extract) was collected. Chromatin-bound extraction buffer was prepared with CaCl₂, micrococcal nuclease, and room temperature NEB and then added to the pellet, vortexed for 15 seconds, incubated in a 37⁰C water bath for 5 minutes, followed by another 15 second vortexing. The sample was centrifuged at the highest centrifuge setting for 5 minutes and the supernatant (chromatin-bound extract) was collected. Room temperature PEB was added to the pellet, vortexed for 15 seconds, and incubated for 10 minutes. The sample was centrifuged at the highest centrifuge setting for 5 minutes and the supernatant (cytoskeletal extract) was collected.

**Total Protein Assay:**

The collected fractions were analyzed using a total protein assay (Bio-Rad). Absorbance was measured at 340 nm for five known standards containing 0.25 µg/µL, 0.5 µg/µL, 0.75 µg/µL, 1.0 µg/µL, and 1.25 µg/µL of bovine serum albumin in 1.5 mL of protein expression buffer to create a standard curve (R² > 0.9). This curve was then used to calculate the concentration of each fraction taken. After the completion of the total protein assay, each fraction was aliquoted and flash-frozen in liquid nitrogen and stored in a -80⁰C freezer for future use.
Western Blot:

The protein extracts from the fractionation experiment were thawed and mixed with a pipette. 10 µg of each extract were diluted and placed into PCR tubes with 15uL of 2X Sample Loading Buffer, keeping the sample to dye ratio 1:1. These samples were denatured by heating them in a boiling water bath for five minutes. Each extract was run on a 12% Mini-Protean TGX Stain-Free Precast gels (Bio-Rad) at 200V until the blue dye reached the bottom of the gel. The Bio-Rad Chem-Doc was used to photograph the gels.

The gels were placed into individual trays and soaked in chilled 1X Western Transfer Buffer, diluted from Bio-Rad 10X Western Transfer Buffer (200mL methanol, 100mL 10X Tris/Glycine buffer, 4g SDS, and 700mL dH2O), for 10 minutes. An Immunoblot PVDF membrane (Bio-Rad) was prepared. The membrane was cut to be the same size as the gels and soaked in methanol until it becomes slightly translucent. The membrane was then soaked in H2O for 3-4 minutes and put on a rotating shaker. The membrane was then soaked in 1X Western Transfer Buffer (Bio-Rad) for 5 minutes. The materials were placed into the transfer cassette as following: sponge, Whatman filter paper, gel, PDVF membrane, Whatman filter paper, sponge. The gel and membrane were placed very carefully so that there were no bubbles and the membrane lined up with the gel. The transfer cassette was placed into an electrophoresis tank and the remaining 1X Western Transfer Buffer was added. The gel was run on a magnetic plate in the cold room at a constant current of 250mA with constant stirring. The membranes were blocked in a sealed tray with a standard blocking solution containing 0.2% nonfat dry milk in 1X Tris-Buffered Saline and Tween 20 (TTBS) buffer solution.
Antibody Detection:

The blocking buffer from the previous procedure was removed from the membrane and the blot was rinsed with TTBS (Bio-Rad) on a rotating shaker two times for 5 minutes each. While the membrane was washed, the primary antibody was prepared on ice using the dilutions listed in Table 1. We used five primary antibodies — a histone 2A (H2A) antibody to detect the nuclear and chromatin proteins, a glyceraldehyde phosphate dehydrogenase (GAPDH) antibody to detect cytoplasmic proteins, a total tau antibody (TAU-13), a 3R tau antibody (3RT), and a 4R tau antibody (4RT). These were utilized to ensure the nuclear fraction was pure and that similar amounts of proteins were loaded into each lane of the gel.

Table 1. Antibodies and dilutions used for detection.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A (Millipore Cat #07-146) Lot #2880714</td>
<td>1:5000 — 1 hour RT 3uL in 15mL TTBS</td>
<td>AP Conjugated Affinity (Millipore Cat. #AP307A) Lot #2912084</td>
<td>1:3000 — Overnight 4°C 5uL in 15mL TTBS</td>
</tr>
<tr>
<td>GAPDH (Millipore Cat. #AB2302) Lot #2967896</td>
<td>1:2000 — 1 hour RT 7.5uL in 15mL TTBS</td>
<td>Chicken — Anti-HRP (Millipore Cat. #AB2302) Lot #2424824</td>
<td>1:12,000 — 1 hour RT 1.25uL in 15mL TTBS</td>
</tr>
<tr>
<td>TAU-13 (Santa Cruz: sc-21796)</td>
<td>1:2000 — 1 hour RT 7.5uL in 15mL TTBS</td>
<td>Mouse — Anti-HRP (Millipore Cat. #AP181P)</td>
<td>1:2,000 — 1 hour RT 7.5uL in 15mL TTBS</td>
</tr>
<tr>
<td>3R Tau (Millipore Cat. #05-803) Lot #2373392</td>
<td>1:3000 — 1 hour RT 7.5uL in 15mL TTBS</td>
<td>Mouse — Anti-HRP (Millipore Cat. #AP181P)</td>
<td>1:2,000 — 1 hour RT 7.5uL in 15mL TTBS</td>
</tr>
<tr>
<td>4R Tau (Millipore Cat. #05-804) Lot #2880714</td>
<td>1:3000 — 1 hour RT 7.5uL in 15mL TTBS</td>
<td>Mouse — Anti-HRP (Millipore Cat. #AP181P)</td>
<td>1:2,000 — 1 hour RT 7.5uL in 15mL TTBS</td>
</tr>
</tbody>
</table>
The final TTBS wash was dumped and approximately 15mL of the primary antibody solution was added to the tray. The membrane and antibody were incubated at room temperature for an hour while on a shaker. The primary antibody was discarded and the membrane was washed multiple times with TTBS while shaking. The secondary antibody was then prepared on ice using the dilutions listed in Table 1. This solution was placed onto the membrane and incubated for one hour at room temperature while shaking. The membrane was washed three times with TTBS for five minutes each. These antibodies were stained with horse-radish peroxidase (HRP) or alkaline phosphatase (AP) and corresponding HRP (Bio-Rad #170-5041) and AP (Bio-Rad Cat. #170-5014) detection kits were used. When using the HRP detection kit, Immun-Star HRP Luminol/Enhancer was mixed with Immun-Star HRP Peroxide Buffer in a 1:1 ratio for a total volume of 2-5mL based on the membrane size. This solution was then added to the membrane and incubated for five minutes. When using the AP detection kit, 2-5mL of Immun-Star Substrate was added to the membrane and incubated for five minutes. In both procedures, excess enhancer solution was removed before the membrane was exposed to light. Chemi-luminescence was detected using a Chemi-doc system (Bio-Rad) and band intensities were determined using the Chemi-doc software package.

**Relative Quantification of Tau Isoforms on Western Blot by Gel Densitometry**

After western blotting was completed, gel densitometry was completed using ImageLab software (Bio-Rad) to quantify the amount of each band detected in the cytoplasm, nucleus, and chromatin relative to the total amount of protein found in all of the fractions. To do this a gel densitometry report was generated by the ImageLab software with volume measurements for the bands in each lane. These volume measurements were all added together to get the total volume density for the membrane. The volume measurement of each individual
band was then compared to the total volume density of the membrane and converted into a percentage of protein detected. See Appendix I for full protocol.

**Band Shift Detection:**

A basic outline for EMSAs was found from Dent and Latchman and Berman *et al.* (1993; 1987). The procedure of detecting band shifts had many changes made to it in order to determine the optimal procedure. The process of forming double stranded DNA, staining/destaining the gel, and detection of bands were standard. All other factors such as DNA amount, protein amount, protein sample content, binding reaction buffer, binding reaction incubation time, loading dye presence, agarose gel concentration, electrophoresis voltage, and electrophoresis time were all varied in different experiments.

To make double stranded DNA, both of the 58 bp forward and reverse strands of the *NOSI* probe are denatured in a PCR cycler at 95°C for three minutes, and annealed at 65°C for one hour. This forms our just DNA control samples (JDC samples).

**Figure 2. The *NOSI* probe.** The top strand shows the forward primer, and the bottom strand shows the reverse primer. The two sequences aligned together as shown form the *NOSI* probe.

The double stranded DNA (5-10 µg) was then mixed with a sample of protein (5-25 µg of nuclear fraction proteins from 5FK or 2FK, or a somewhat purified tau sample) and binding reaction buffer (PBS (1X PBS, pH 7.4), Tris (20 mM Tris-HCl, pH 7.2-7.6), or HEPES (10 mM HEPES, 12.5% glycerol, 50 mM KCl, 12mM MgCl₂ pH 7.5)) to a total volume of 18 uL for PBS or Tris buffers, or 24 uL for HEPES buffers (MSA samples). The MSA samples were incubated at
4°C (achieved by either incubating on ice or in the 4°C cold room) for 30-90 minutes. When Tris or PBS buffer was used, the samples needed to be mixed with 4X loading dye to a final total volume of 24 uL; Hepes buffer did not require the addition of loading dye. All samples were loaded on to a large 2-3% (w/v) agarose gel and run at 30-100 V in 1X TAE for 5-24 hours. When agarose concentrations above 2% were used, the agarose was left in 1X TAE buffer for 15 minutes prior to a very slow heating process, avoiding boiling of the solution.

To perform a secondary shift, either the normal protein sample was changed to a more purified sample of tau, or an antibody was added to the MSA sample incubation mixture. To obtain a more purified sample of tau, Barghorn et al. outlines a purification from whole-cell lysates involving boiling the sampling for 20 minutes with 0.5M NaCl and centrifuging at 127,000g for 40 minutes at 4°C, which removed nearly all proteins of lower molecular weight than tau (2005). When adding an antibody to the reaction mixture, the NOS1 probe and protein sample must incubate without the antibody for 30 minutes, then the 1 µg of antibody may be added. Each antibody useful in these secondary shift EMSAs existed at different concentrations, and thus different volumes were used accordingly: 5 µL of total tau antibody = roughly 1 µg, 10 µL of 3R tau antibody = 1 µg, 1 µL of GAPDH antibody = 1 µg. Appropriate controls must also be added to an EMSA which utilized an antibody. A normal JDC and MSA sample were run next to the MSA sample containing antibody, as well as a JDC sample with the same amount of antibody and no protein, and another MSA sample with the same amount of an unrelated antibody, such as the one for GAPDH.

The final gel was stained with a 0.5 µg/ml ethidium bromide solution for 15 minutes and destained in dH2O for 5 minutes. The Bio-Rad Chem Doc was used to photograph the gel to detect the bands.
RESULTS

Western Blot and Antibody Detection:

Western blotting was completed with antibodies specific to different tau isoforms to identify which isoforms are found in different subcellular fractions. We obtained a total of two fractions using the 2FK fractionation kit: cytoplasmic and nuclear; and five fractions using the 5FK fractionation kit: cytoplasmic, membrane, soluble nuclear, chromatin-bound, and cytoskeletal. Each fraction was ran on a gel and it was confirmed that there was some functional protein present in each fraction (Figure 3).

![Gel analysis displaying the presence of protein in all fraction samples.](image)

**Figure 3. Gel analysis displaying the presence of protein in all fraction samples.** Approximately 10μg of protein from the cytoplasmic (CY), chromatin (CH), and nuclear (N) fractions were run on a 12% Mini-Protean TGX Stain-Free Precast gels (Bio-Rad) at 200V. Presence of similar amounts of protein was observed in each fraction, allowing us to proceed to a western blot analysis.
We determined that the 2FK fractionation protocol yielded the expected fractions, using GAPDH as a marker for the cytoplasmic fraction and histone H2A as a marker for the nuclear fraction. A strong positive band of 38 kDa is present in the cytoplasmic fraction for GAPDH, and a very faint potential band of 17 kDa was detected in the nuclear fraction for H2A (Figure 4A).

**Figure 4. Antibody analyses of two subcellular fractions from SK-N-MC cells.** Approximately 10 µg of DNA in both fractions was loaded onto a gel and then transferred onto a PVDF membrane. (A) Relative purity of the cytoplasmic (CY) and nuclear (N) fractions were confirmed using GAPDH and H2A antibodies. (B) Presence or absence of the tau isoforms located in the two fractions after exposure to Total Tau (TAU-13) antibody. (C) Presence or absence of the 3R and 4R tau isoforms distributed in both fractions.

Next, we determined the distribution of the Total tau, the 3R tau, and the 4R tau isoforms in these fractions (Figure 4B, 4C). Analysis using an antibody against all tau isoforms (TAU-13) revealed that numerous tau isoforms were present in the cytoplasm and nucleus, showing bands between 35 and 50 kDa that appear to be equal strength (Figure 4B). When a 3R tau antibody was used for detection, similar detection patterns were observed in the cytoplasmic and nuclear fractions, but the amount of tau appeared to be greater in the cytoplasmic fraction (Figure 4C).
These bands were detected at 110 kDa and between 35 kDa and 45 kDa. Little tau expression was observed when the 4R tau antibody was used. Faint bands were detected at 20 kDa and 46 kDa (Figure 4C).

We then examined whether the 5FK fractionation protocol yielded the expected fractions, using GAPDH as a marker for the cytoplasmic fraction and histone H2A as a marker for the nuclear and chromatin-bound fraction. A strong positive band of 38 kDa is present in the cytoplasmic fraction for GAPDH, while less intense bands were found in the nuclear and chromatin fractions at approximately 17 kDa for H2A (Figure 5A).

![Figure 5. Antibody analyses of five subcellular fractions from SK-N-MC cells.](image)

Approximately 10 µg of DNA in each fraction was loaded onto a gel and then transferred onto a PVDF membrane. (A) Relative purity of the cytoplasmic (CY), nuclear (N), and chromatin-bound (CH) fractions were confirmed using GAPDH and H2A antibodies. (B) Presence or absence of the tau isoforms located in the three fractions after exposure to the TAU-13 antibody. (C) Presence or absence of the 3R and 4R tau isoforms distributed in the three fractions.
Next, we investigated the distribution of the Total tau, the 3R tau, and the 4R tau isoforms in these fractions (Figure 5B, 5C). Analysis using an antibody against all tau isoforms (TAU-13) revealed that numerous tau isoforms were present in the cytoplasmic, chromatin, and nuclear fractions. The bands ranged in size between 35 kDa and 50 kDa (Figure 5B). Bands of similar sizes were found in all examined areas of the cell. The amount of tau was found to be greater in the cytoplasm, next greatest in the chromatin, and lowest in the nucleus (Figure 5B). A band indicating the Big Tau isoform was detected at approximately 110 kDa in all three fractions. When a 3R tau antibody was used for detection, similar detection patterns were observed in the cytoplasmic and chromatin fractions. Bands were detected between 35 kDa and 45 kDa (Figure 5C). Very little tau expression was observed when the 4R tau antibody was used; however, faint bands may be detected at approximately 20 kDa and 46 kDa (Figure 5C).

**Relative Quantification of Tau Isoforms on Western Blot by Gel Densitometry**

Following antibody detection, a more quantified view of our results was seen as necessary to help understand the amount of each tau isoform in the cytoplasmic, nuclear, and chromatin fractions of the cell and to accurately compare these amounts to each other. Gel densitometry of membranes exposed to both a 3R tau antibody and a total tau antibody demonstrated low amounts of nuclear tau in SK-N-MC cells (Figure 6). Relative quantification of tau isoforms detected on a western blot exposed to a total tau antibody also demonstrated an increased amount of tau in the cytoplasm and chromatin fractions, adding up to be 83.34% of protein detected (Figure 6A). It was found that the tau isoform in “Band 1” predominates in the cytoplasmic fraction, measuring at 13.76% of total protein. It was also observed that the tau isoform detected in “Band 4” predominates in these fractions, measuring to be a total of 28.73% of protein on the membrane.
Figure 6. Relative quantification of tau isoforms by gel densitometry. Image Lab software (Bio-Rad) was used to quantify the amount of each tau isoform detected in the cytoplasm, nucleus, and chromatin of SK-N-MC cells. Percentages of protein in each fraction were calculated by comparing each band’s volume to the entire volume of bands in all three lanes. A) Relative quantification of proteins detected using a Total Tau antibody. B) Relative quantification of proteins detected using a 3R tau antibody.

Relative quantification of proteins detected when a 3R tau antibody was used demonstrated an increased amount of 3R tau in the cytoplasm and chromatin of SK-N-MC cells, adding up to be 48.27% protein in the cytoplasm and 41.75% protein in the chromatin (Figure 6B). Again, it was found that the tau isoform represented as “Band 1” predominates in the cell. Out of the remaining bands detected using a 3R antibody, it was observed that “Band 3” predominated in these fractions, adding up to be 20.06% of total protein.
Figure 7. Overlay of 3R tau antibody detection on total tau antibody detection. Imaging software was used to overlap the bands detected using both the 3R tau antibody and the Total tau antibody. The blue bands represent the 3R tau and the grey represents the Total tau.

Patterns observed for Total Tau and 3R Tau in the cytoplasmic and chromatin fractions were very similar, but there were more bands detected when using a Total Tau antibody, suggesting that 4R isoforms are present (Figure 7). Using this overlay along with the relative quantifications of tau protein in each fraction, and the known molecular weights, we were able to identify which tau isoforms were detected in each fraction on the membranes (Table 2).
Table 2. Likely identifications of tau isoforms and detected bands on western blots.

<table>
<thead>
<tr>
<th>Total Tau Antibody</th>
<th>3R Tau Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1</td>
<td>Big Tau</td>
</tr>
<tr>
<td>Band 2</td>
<td>4R Tau Isoform</td>
</tr>
<tr>
<td>Band 3</td>
<td>2N3R</td>
</tr>
<tr>
<td>Band 4</td>
<td>1N3R</td>
</tr>
<tr>
<td>Band 5</td>
<td>0N3R</td>
</tr>
</tbody>
</table>

Electrophoretic Band Shift Assays:

Our lab has previously linked tau protein presence with changes in *NOS1* expression, though the mechanism by which this change in expression occurs is unknown. We designed EMSAs in order to determine if direct DNA binding by tau or another nuclear protein is the cause of this change in *NOS1* expression. The first step in creating an EMSA is to ensure an effective probe is utilized. This was investigated in Figure 8, visualizing the single the forward reverse strands for the *NOS1* probe on an agarose gel. Lanes 1 and 3 contain the forward and reverse primers annealed together, forming the *NOS1* probe. All lanes contain total 8.1 µg of DNA and are visualized on a 2% (w/v) agarose gel.
primers, respectively, and show far dimmer bands seen slightly above the band seen in lane 2. Lane 2 shows a very vivid band which contains both single strands after the annealing procedure, creating the *NOS1* probe (Figure 8).

Next, we began to visualize our probe when mixed with nuclear proteins. When highly degraded proteins are used in MSA samples, a complete lack of shifting is seen and can be utilized as a negative control (Figure 9A). An accompanying polyacrylamide gel run with the same protein samples showed no visible bands, indicating that all proteins had completely degraded (data not shown). However, all EMSAs run with viable proteins (confirmed through visualization on a polyacrylamide gel, data not shown) showed MSA samples displaying a change in mobility. A representative figure is seen as Figure 9B.

**Figure 9. Non-denatured nuclear proteins slightly shift the NOS1 probe.** For both (A) and (B), lane 1 contains only the NOS1 probe. Lane A2 contains highly degraded nuclear proteins in Hepes buffer while lane B2 contains non-denatured nuclear proteins in Tris buffer. All samples were visualized on a 2% (w/v) agarose gel.

Several EMSAs were conducted under varying conditions as described in the methods to create the most effective EMSA. In one EMSA comparing incubation time and buffer, all MSA samples showed a change in mobility with the exception of lane A2. Lane B3 also appeared
dimmer than all other bands. No difference was seen between samples which had incubated for 30 minutes (lane 2) and 90 minutes (lanes 3 and 4) (Figure 10).

Figure 10. Investigating the effects of buffer type and incubation time on EMSAs. All samples were resolved on a 2% (w/v) agarose gel. Samples in lanes 1 and 5 are JDC and lanes 2-4 contain MSA samples using nuclear proteins (5FK, 21 µg). Lane 2 contains samples incubated for 30 minutes, while samples in lanes 3 and 4 were incubated for 90 minutes. A) Samples were run after incubation in PBS buffer. B) Samples were run after incubation in Tris buffer.
DISCUSSION

Overall Discussion:

The overall goal of this project was to further characterize tau’s presence and function in the nuclei of human cells. After fractionation of SK-N-MC cells, tau isoform presence was determined through western blotting and antibody analysis, and a potential DNA-binding role for tau was explored using EMSAs.

Prior to exploring either aim, a reliable set of compartmentalized proteins was necessary. A major obstacle in obtaining these reliable proteins was sufficient compartmentalization. We found that in order to obtain well compartmentalized fractions, it was critical for the cell pellet to be left completely dry during each fractionation step before the addition of the following buffer solutions. This was accomplished through the second, short centrifugation after the removal of each fraction from the pellet as described in methods. This was done to ensure all liquid had been removed, and if it had not then we were able to remove it. Successful compartmentalization was achieved in Figure 4A and Figure 5A. In both analyses, the expected bands were detected when antibodies for GAPDH in the cytoplasm and H2A in the chromatin and nuclear fractions. This showed that acceptable fractionation occurred, with a large amount of GAPDH in the cytoplasm and very little in the nucleus and chromatin, and H2A only being present in the nucleus and chromatin.

Throughout this project, protein degradation resulted in the largest reason for failure in obtaining results. The importance of maintaining viable proteins can be visualized in Figure 9, where a difference in mobility is seen when the sample contains known viable proteins and no difference in mobility is seen for a sample containing fully degraded proteins. The viable proteins were utilized in the pictured EMSA after roughly two weeks of storage, and the EMSA
containing degraded proteins was run after three weeks of storage (Figure 9). Another EMSA run with both Tris and Hepes incubation buffers after proteins had been stored for 7 weeks showed very slight changes in mobility with no difference between the incubation buffers, despite the samples in Tris buffer being treated exactly as they had been in Figure 10B (data not shown). Thus, a clear relationship between length of time stored and protein viability was not seen, but instead the treatment of the fractions prior to flash freezing was likely what led to degradation. Each fractionation sample was kept on ice until completion of the total protein assay, but if samples sat on ice for too long or extra care was not taken for the aliquot tubes to be made cold before the addition of each sample, degradation likely began prior to freezing.

**Aim 1:**

The goal of this aim was to determine the isoforms of tau normally found in the nucleus of human SK-N-MC cells. This was examined through the analysis of western blots using antibodies for total tau (TAU-13), 3R tau, and 4R tau.

Initial western blot analyses resulted in low intensity bands with a significant amount of background (data not shown). Switching secondary antibodies to one that contained horseradish peroxidase resulted in less background noise in comparison to using an alkaline phosphatase detection kit. In order to obtain stronger bands, varied antibody incubation times were used. We found that for the GAPDH, total tau (TAU-13), 3R tau, and 4R tau antibodies, one hour incubations at room temperature allowed for better detection. The best results with the H2A antibody were observed with overnight incubation at 4°C.

Although tau has primarily been described as a microtubule-associated protein with a preferential axonal localization, it has been observed in the nuclei and chromatin of human neuronal cells (Liu and Gotz, 2013). Antibody detection of the 2FK cytoplasmic and nuclear
fractions detected similar patterns of bands in these two fractions. When using the total tau (TAU-13) antibody, the same band pattern and intensity was observed in the cytoplasm and nuclear fractions. Detection with the 3R tau antibody resulted in similar bands in both fractions with an increased intensity in the cytoplasm, which, like in the 5FK proteins, suggests that while the same 3R isoforms are present, there is more 3R tau in the cytoplasm. We believe that this lesser prevalence of tau in the 2FK nuclear fraction is due to more of the nuclear tau being bound to DNA and thus not collected in this fraction. Comparison of the intensities of 2FK and 5FK cytoplasmic and nuclear fractions, respectively, appear almost identical, supporting this claim (Figures 4C, 5C).

Western blot analysis of the fractions obtained using the 5FK kit detected similar band distributions in the cytoplasm, nucleus, and chromatin of SK-N-MC cells when treated with Total Tau and 3R tau antibodies (Figure 5). The observed pattern in the nucleus was slightly less intense when compared with the cytoplasm and chromatin. These results suggest that the same tau isoforms are located in the cytoplasm, nucleus, and chromatin of neuronal cells, and also that the majority of nuclear tau binds to DNA instead of remaining free in the nucleus. This is in agreement with similar studies in mice (Liu and Gotz, 2013).

Relative quantification of tau isoforms on western blots was measured using gel densitometry (Figure 6). Based on these results and the isoform identifications in Table 2, the Big Tau isoform had the greatest percentage of detected protein in the cytoplasmic fractions (Figure 6). This suggests that Big Tau does not have a high binding affinity for DNA or free-floating nuclear tau. Higher percentages of tau protein in Band 4 of the membrane exposed to a total tau antibody and Band 3 of the membrane exposed to a 3R tau antibody suggest that the 1N3R tau isoform is more predominate in the cell. This is interesting because the 1N4R isoform
was found to predominate in studies of mouse neurons (Liu, Gotz, 2013). The increased percentages of tau in the chromatin compared to the nucleus also suggests that a majority of nuclear tau is found to be bound to DNA, rather than remaining free in the nucleus. Most of the small tau isoforms also seemed to be present in the cytoplasmic, nuclear, and chromatin fractions (Figure 6). Further experimentation should be done to quantify the relative amounts of 4R tau isoforms in the nuclear and chromatin fractions to investigate the ratio of 3R tau to 4R tau in these cellular fractions.

When exposure to both the total tau and the 3R tau antibody occurred, presence of the Big Tau isoform was detected. Research completed on the UCSC Genome Browser by Dr. Raymond Enke in the JMU Biology Department suggests that there is an increased amount of Big Tau in human SK-N-MC cells (Figure 11B). Increased big tau expression in SK-N-MC cells is thus noted at both the RNA and protein levels.

Detection of 4R tau showed very little 4R tau was present in subcellular fractions from both fractionation kits (Figures 4C, 5C). Faint detection of 4R tau may be due in part to the quality of the 4R antibody as bands undetected by the 3R or 4R antibody were found in the total tau fractions (Figure 7). Moreover, UCSC Genome Browser RNA-sequencing by Dr. Raymond Enke illustrates a lower presence of 4R tau RNA in SK-N-MC cells in comparison to what is observed in the human brain (Figure 11C). While this would contribute to a higher difficulty of detection, we also believe our low detection was a result of the 4R tau antibody being of poor quality or the antibody degrading. Protein degradation may have occurred when separating the antibody into several aliquots. Similar studies have observed higher amounts of mature 4R tau in the SH-5Y-SY cell line (Smith et al). Further experimentation should be completed to investigate the amount of 4R tau in other neuronal-like mammalian tissue culture cells.
Figure 11. UCSC Genome Browser research from Dr. Raymond Enke showing RNA-sequencing data comparing human brain RNA and SK-N-MC cells. In Fall 2017, Dr. Raymond Enke completed research on the UCSC Genome Browser. A) RNA-sequencing in human brain cells and SK-N-MC cells shows RNA transcripts as peaks, green and red respectively. The full transcript of the MAPT gene allows for comparison of exon expression in human brain RNA and SK-N-MC cells. Differing peak heights likely reflect differences in expression. B) It is of particular note that SK-N-MC cells appear to contain more exon 4A transcripts suggesting they have more Big Tau isoform.* C) It is of particular note that SK-N-MC cells contain very little exon 10 transcripts suggesting they have little 4R tau isoform.*
The results of this aim suggest that there is similar distribution of tau isoforms in the cytoplasm, chromatin, and nucleus. It was found that both the 2FK and 5FK kits may be used to collect proteins, though in both cases the nuclear fraction contains a lesser amount of the same isoforms of tau. Gel densitometry of protein bands suggests that the Big Tau and 1N3R tau isoforms predominate in the cytoplasm of SK-N-MC cells. These findings suggests that a majority of nuclear tau may be bound to DNA, rather than free in the nucleus, further supporting tau’s potential role as a DNA binding factor.

Aim 2:

The goal of this aim was to learn how tau or another agent could regulate NOS1 gene expression through direct DNA binding. This was investigated through the development of effective EMSAs, with positive results obtained from creating the NOS1 probe and an EMSA varying incubation time and buffer type.

The formation of the NOS1 probe used in each EMSA was effective. Figure 8 shows the two single stranded pieces as far less intense bands appearing higher on the gel than the two strands annealed together. We expected to see the bands of both single strands lower on the gel as these are smaller molecules than double stranded molecules, however secondary structures may have formed from the single strands folding over on each other to cause a more irregular structure with lower mobility. To visualize the DNA, the gel was stained using ethidium bromide, a DNA intercalating agent, which fluoresces far brighter when inserted in between double stranded DNA than when associating with single stranded DNA (Fuller and Waring, 1964). This property of ethidium bromide explains why the bands of single stranded pieces appeared far dimmer and what we believed to be a double stranded piece of DNA appear far
brighter. The band in lane 2 being so much brighter than either lane 1 or 3 determined that our annealing procedure was effective (Figure 9).

The EMSA which varied incubation time and buffer showed no difference in mobility for all MSA samples, with the exception of lane A2 (Figure 10). Lane B3 also showed a far dimmer band than all others, likely due simply to a mistake in pipetting into the gel. The absence of change in mobility for the sample in lane A2 can be explained by an insufficient amount of protein mixed with the sample. While mixing DNA and protein samples, this was the last sample mixed and received the last of the nuclear proteins we had collected and ended up being less than the total amount of proteins in other samples (Figure 10). This lack of a similar amount of total protein leading to a lack of shifting also gives support to our observations of degraded proteins leading to a lack of shifting. It is clear that at least 20 µg of total nuclear proteins is necessary to visualize an effective shift of the \textit{NOSI} probe. The requirement of a large amount of total protein may suggest that the protein causing the shift of the \textit{NOSI} probe is at a low concentration, or that multiple proteins are necessary to cause the shift of the \textit{NOSI} probe. Future experiments are necessary to further investigate the agent(s) causing the shift of the \textit{NOSI} probe.

In all EMSAs performed, a large amount of streaking was seen, both leading up to the band from the loading wells and a small amount following after the band, and the band was quite large and not very distinct. The presence of streaking in every lane, not just those with proteins mixed, leads us to believe that the streaking is from the \textit{NOSI} probe, not anything to do with the proteins that had been mixed with the \textit{NOSI} probe. We believe that streaking may be minimized through using less total DNA in our EMSAs. In one attempted EMSA which utilized Hepes buffer, a lesser amount of total DNA was used (5 µg, as opposed to 8.1 µg in previous EMSAs) and did not show any visible effect on mobility, streaking, or band size (data not shown). We
believe in future studies, even less than 5 µg of DNA, potentially as low as 500 ng of DNA, should be attempted, as this may lead to a more distinct band with less streaking.

Useful information for developing our EMSA technique was found each time an EMSA was attempted, even in those not presented as figures. In an EMSA previously mentioned, Hepes buffer appeared to be just as effective as the Tris buffer. In this same EMSA, both 5FK and 2FK fractions were used, and some lanes contained less of the nuclear fraction (some lanes contained 15 µg and others contained the same as past EMSAs—20 µg). No difference was seen when proteins from either fractionation kit was used, however, when a lower amount of protein was used a smaller shift was observed. This EMSA was not presented as a figure due to the fact that shifts were barely seen, and thus the discussion of results from this EMSA are considered preliminary and require repetition for verification (data not shown). Using Hepes buffer also allowed for investigation in EMSA runtimes. Several different voltages and their resulting different runtimes were utilized with various EMSAs, and while distance traveled by the samples increased resolution and allowed mobility changes to be seen, no relationship was observed between runtime and magnitude of mobility change. Thus, any voltage can be used to visualize changes in mobility as long as the samples are run to the end of the gel. The greatest concern with higher voltages was that the running buffer and gel would warm and cause the DNA-protein interaction to break, or begin degrading the proteins entirely. Voltages even up to 120 V were seen to not warm the running buffer or gel as long as the apparatus was kept in the 4°C cold room for the duration of the run (data not shown). Longer runtimes (>10 hours) were found to only be possible when our Hepes was used as the incubation buffer. All samples require a greater density than the running buffer in order to make sure that the sample remains in the gel and does not diffuse into the running buffer. All incubation buffers besides Hepes required mixing with
loading dye to give the sample its required density, but Hepes contained a much higher concentration of glycerol, giving it a far greater density and allowing it to remain in the gel for longer. Samples mixed with loading dye were observed to diffuse out after 10 hours, but samples mixed with the Hepes buffer were able to remain in the agarose gel for at least 4 days without losing any sample (data not shown).

Other variables attempted to observe changes in mobility included varying protein amount from 5-25 µg, varying MgCl₂ concentration from 1-20 mM in the incubation buffer, using cytoplasmic proteins as opposed to nuclear proteins, and attempting a supershift with our total tau antibody (TAU-13). The EMSA varying protein amount was intended to confirm our observation that protein amount seems to be the largest factor in the amount of mobility change seen. Varying the concentration of MgCl₂ was to be the first of several EMSAs investigating the ideal incubation buffer for observing a change in the NOSI probe’s mobility. MgCl₂ is an important buffer ingredient for our investigations as the concentration of MgCl₂ can change the structure of R/Y repeat sequences of DNA from B-DNA to Z-DNA. Lower salt concentrations can create an environment in which the B-DNA predominates, and higher salt concentrations can form an environment in which the Z-DNA form predominates (Bothe et al., 2011). Tau is known to have an affinity towards both forms, however if tau is shown to bind this sequence in the Z-DNA form that could more strongly connect it into the narrative of tau causing the excess of Z-DNA seen in AD brains by stabilizing many of the genes related to AD with R/Y repeats into Z-DNA. The use of cytoplasmic proteins in an EMSA could have provided some indirect evidence that tau is binding to the NOSI probe as it is found in roughly equal amounts in the cytoplasm and nucleus (Figure 5). Finally, a supershift could have confirmed or denied tau’s involvement in
the shifting of the \textit{NOSI} probe. Unfortunately, no results were found in all of these attempts due to protein degradation (data not shown).

Future EMSAs should explore the aforementioned parameters for which results were not obtained due to protein degradation, as well as repeat the EMSA involving the Hepes buffer to verify the conclusions drawn from that data, continue varying other incubation buffer pH and ingredients (DNA amount, protein amount, concentration of KCl, addition of a non-competitive DNA binder), an attempt at a crude purification of tau should be made to see if mobility shifts improve with a fewer number of total proteins, and finally a fully purified sample of tau or use of the total tau antibody must be completed in order to determine if this is the agent binding the \textit{NOSI} probe.

It is very possible that future EMSAs may show tau is not directly binding to the \textit{NOSI} probe, or that multiple molecules, including tau or not including tau, are binding to the \textit{NOSI} probe. Any result, whether it is tau or not binding the \textit{NOSI} probe, would still be very important in understanding why the R/Y repeat of \textit{NOSI} is required for a change in expression to occur due to tau’s presence. If a molecule besides tau was found to be binding the \textit{NOSI} probe, this may determine a completely new role for tau in the nucleus as regulating some unknown nuclear protein, furthering our knowledge of tau in the nuclei of neurons. The identity of that protein could also be determined through EMSAs using various purified nuclear samples. There is still far more room for discovery in this area.

From our successful EMSAs, we have learned that something in the nuclear fraction of SK-N-MC cells is capable of causing a slight shift in the \textit{NOSI} probe, suggesting direct DNA binding, though we have not identified this protein (Figure 8). We have also learned that there is
not a great difference seen between the Tris and PBS buffers, and sample incubation can occur for only 30 minutes and effective shifts are still seen (Figure 9).

**Final Conclusions:**

The goal of this project, to further investigate tau’s presence and function in the nucleus of human neuronal cells, was successfully accomplished. We determined that the same 3R isoforms are present in the cytoplasm, nucleus, and chromatin of SK-N-MC cells. It was also found in Aim 1 that a large majority of nuclear tau is found to be bound to DNA, as especially the 1N3R isoform, rather than remaining free in the nucleus, providing new evidence to support tau’s role as a DNA binding factor. And, in Aim 2, advances were made in the development of methods to investigate tau’s direct binding ability to a common repeat found in the promoters of many genes involved in Alzheimer’s.

While future studies directly related to each specific aim have already been stated, there are many other studies to further our knowledge of tau in the nucleus that do not pertain specifically to either aim. As demonstrated in our results, the Big Tau isoform predominates in the cytoplasm of non-neuronal cells. Although many studies have not been done on the role of this isoform, further experimentation should be done to identify the role of the extra exons present in Big Tau and understand how they may keep this form of tau out of the nucleus. A common practice in order to determine the function of a protein is to create a knockout cell line missing the gene for the desired protein. Experiments have already begun in our lab utilizing the CRIPSRT-Cas9 system in order to obtain SK-N-MC knockouts for various isoforms of tau. These cells would allow for a large variety of experiments to determine what tau could normally be doing, such as analyzing differences in expression of several genes involved in Alzheimer’s between tau knockout cells and wild-type cells.
APPENDIX I. Gel densitometry protocol adapted from the Bio-Rad Image Lab software user guide.

First, open the image of the exposed membrane in the Image Lab software. With your image open in the workspace, click Lane and Bands in the Analysis Toolbox located on the left panel.

To detect lanes…

1. To detect a specific number of lanes, click Manual and enter the number of lanes you want to find.
2. Lanes may be moved around to better fit what is detected on your membrane. To move one or more selected lanes, place the pointer on a selected lane and drag it to a new position. If you select multiple lanes at once, they will all move together.
3. To resize a lane, first select the lane you want to edit and white anchor points will become visible. Point to an anchor on the side of the lane and drag the anchor to change the lane width to your liking. To resize lanes to a width you specify, in the lanes tab enter a width in millimeters and click Set Width.

To detect bands…

1. First, click “Detect Bands” in the Lane and Bands setting in the Analysis Toolbox.
2. To manually add a band to a lane, use the “Add” feature in the Bands tab. First, click add and then click inside the lane where you would like to define a band.
3. To delete a band, click delete on the Bands tab and then click the band you want to remove.
4. You may use the adjust feature to display boundary lines above and below each band. Move the pointer over a boundary line until the cursor changes to a double-pointed
arrow. Move the boundary line to adjust the band height. Image Lab will reposition the band and recalculate its center.

Once your desired number of lanes and bands have been defined, click the “Report” on the toolbar. This will create a file including your membrane image with the defined lanes and bands along with a series of tables containing the volume measurements of each band.

To calculate relative percentage of protein detected in each band…

1. First make sure to record each volume measurement calculated in the Report from Image Lab.

2. Add together the volume measurements of all bands detected on your membrane.

   Divide each individual band volume by the total volume of protein detected on the membrane and multiply this by 100 to make it a percentage.


Weaver, T., and Deal, A. (2016). Modulation of Nitric Oxide Synthase I Transcription by Tau and Alpha-Synuclein and its Relevance to Alzheimer’s and Parkinson’s Diseases. James Madison University.