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Cross-Seeding Potential of Misfolded Amyloid-beta Strains on Tau Protein: Examining the Puzzle Pieces of Alzheimer's Disease

Sara Kelley

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Cross-Seeding Potential of Misfolded Amyloid-beta Strains on Tau Protein: Examining
the Puzzle Pieces of Alzheimer's Disease

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Cross-Seeding Potential of Misfolded Amyloid-beta Strains on Tau Protein: Examining
the Puzzle Pieces of Alzheimer's Disease

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Sara Elizabeth Kelley, B.S.

Houston, Texas

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Cross-Seeding Potential of Misfolded Amyloid-beta Strains on Tau Protein: Examining the Puzzle Pieces of Alzheimer's Disease

Sara Elizabeth Kelley, B.S.

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Alzheimer's disease (AD) is a neurodegenerative disorder that will progressively impair cognitive function. AD is the leading source of dementia, and affects one third of the senior citizens in the United States. Aging is the primary risk factor for AD and due to the expected rise in human lifespan worldwide, the occurrence rate of AD diagnosis is projected to increase. This observation is increasingly alarming when considering there is no treatment for reversal or prevention of AD yet.

AD is characterized by gross pathological hallmarks in the brain. These include the extracellular accumulation of misfolded amyloid-beta ($A\beta$) and the intraneuronal buildup of tau neurofibrillary tangles (NFTs). Compelling evidence suggests that $A\beta$ triggers tau accumulation. Both of these disease-associated proteins are known to contribute to the typical AD associated clinical signs. Interestingly, these proteins have been shown to display several features similar to those seen in infectious prions, such as their conformational strain diversity. Prion strains trigger diseases that may substantially vary in their pathological and clinical aspects. One example of strain diversity in $A\beta$ can be found in the synthetic 2F and 3F fibrils. These aggregates were originally generated by Petkova *et al.* by modifying protein aggregation protocols. These strains were thoroughly characterized by their structural features, convincingly showing that misfolded $A\beta$ can

adopt different conformations. This paper explores A β seed strains differentially influencing the conformation of tau aggregates in order to promote altered tau pathology.

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Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder that will progressively impair cognitive function such as memory, learning, and reasoning. These commonly described clinical symptoms are due to particular pathological changes in the brain, including the deposition of misfolded proteins, inflammation, synaptic loss and neuronal death (1). The leading risk factor for AD is age, and considering the progressive aging of the world's population, the number of people affected by AD is estimated to reach 13.2 million by 2050 (2).

In 2020, over 6 million Americans were diagnosed with AD, and this number is expected to increase in the coming decades due to the average age of the population increasing (3). The main pathological hallmarks of AD include the aggregation and subsequent deposition of amyloid-beta ($A\beta$) plaques extracellularly, as well as the hyperphosphorylation and deposition of tau tangles intracellularly (4). This process will lead to neuroinflammation, neuronal death and glial activation associated with AD. All of this together positions AD as one of the main public health challenges in the near future (5). Especially considering that there is still no cure for AD, so it is imperative that the mechanisms of the disease are better understood (6).

The most extreme effects of AD can be seen in the entorhinal cortex and hippocampus (7). In a typical brain, there will be shrinkage seen with aging, but this is not usually linked to a large loss of neurons (8). However, in the brains of AD patients there is large, widespread damage to the neurons. Many neurons lose their synaptic connections, stop functioning or die. This process typically begins with the parts of the brain associated

with memory such as the hippocampus and entorhinal cortex. AD spreads from these areas to the region affecting language, social behavior, and reasoning; the cerebral cortex. This will eventually progress to damage even more areas of the brain before ending in fatality (9,10).

The neurological pathology of AD is defined by extracellular amyloid beta ($A\beta$) deposits and intracellular tau aggregates existing in conjunction (11). The pathological presentation of AD begins with the $A\beta$ protein. This protein is unique to AD and is a hallmark of the disease (12). $A\beta$ is formed through the degradation of a larger protein called the amyloid precursor protein (APP). It typically functions in the brain as an essential component of cell growth and repair. The misfolding of this protein, that is linked to familial AD, is seen in a mutation in the genes PS1 and PS2, presenilin-1 and -2, as well as APP (13,14). This mutation has a link to age, to modify its time of onset. It will increase the levels of $A\beta_{42}$ within the brain. When in the brain of an AD patient, these proteins are misfolded and present in abnormally high levels and then clump together in the formation of plaques. These plaques form between neurons and act to disrupt the function of these neurons (15). The other protein associated with AD pathology is tau. Tau proteins are generated through the splicing of the gene MAPT (microtubule-associated protein tau) (16, 17). The main biological function of tau is the promotion of the assembly of tubulin into microtubules. Also, tau is a phosphoprotein, therefore its binding to microtubules is regulated by its phosphorylation (18). When tau becomes misfolded and is hyperphosphorylated it will aggregate into neurofibrillary tangles (NFTs) intracellularly (19). With the presence of $A\beta$ plaques and tau tangles there will be neuroinflammation,

which will lead to neurotoxicity. This will result in neuronal death and synaptic dysfunction (20). Once all of this is active within the brain the patient can be diagnosed with AD. The pathological progression of AD can begin decades before a patient is diagnosed by a clinic (21).

While there are some consistent features of the disease, Alzheimer's disease is clinically and pathologically variable (22). Clinical manifestations of the disease can vary in many ways, such as, the age of onset, the degree and rate of cognitive decline, and the duration of the disease (23). In reference to pathology, differences have been seen in the atrophy of patients' brains through imaging. Dissimilarities have also been seen in plaque morphology, and interestingly there have been patients who have presented with plaques however they did not present clinically with AD (24).

A β aggregates in AD brains can be found in a variety of arrangements, including intracellular aggregates, diffuse plaques, vascular deposits, soluble A β oligomers, dense-core senile plaques, as well as many others. Tau is known to have variation in its conformation among the diseases it is associated with. These diseases also have differences in their clinical presentation depend on the tau isoform they present (25). This variability in the pathology of the two key proteins associated with AD, A β and tau, could possibly be the key to as to why there are differences in the clinical manifestations of AD.

The current, prevailing theory for the progression of AD is the amyloid cascade hypothesis. This theory suggests that the A β deposition on the patient's brain is the crucial step for AD progression (26). Conceptually, this has been the guide for most of the research surrounding AD, for the past 2 decades, both clinically and academically (27). However,

all of the studies that are using this hypothesis in order to reduce A β aggregation or production have resulted in failure in the Phase III clinical trials. This has led the field to re-evaluate the amyloid cascade hypothesis and its relevance when looking to prevent or reverse AD progression (28).

Prion-like transmission of misfolded proteins.

Due to the typical deposition of misfolded forms of A β and tau in the AD brain, this disease can be classified as a Protein Misfolding Disorder (PMD) (29). PMDs are associated with a multitude of proteins including, but not limited to, A β and tau in AD, α -synuclein (α -syn) in Parkinson's disease (PD), misfolded prion protein (PrP^{Sc}) in prion diseases (30). In addition to their intrinsic toxicity, protein aggregates in PMDs chronically activate glial cells within the brain. They are also able to disrupt synaptic signaling and interfere with proteostasis (31). All these events act in a synergistic manner, damaging the brain, inducing clinical signs and invariably leading to the death of the individual (32).

All PMDs have been described with either familial/genetic or spontaneous etiologies. However, one sub-group of PMDs, prion diseases, can also be induced by infection. The mechanisms of protein misfolding and spread in prion diseases has been well studied. In fact, the infectious etiology of prions has been instrumental to understand the biology of misfolded proteins and evaluate diagnostic and therapeutic strategies for all PMDs (33).

Prion diseases, which are also known as transmissible spongiform encephalopathies (TSEs), are a collection of fatal, neurodegenerative diseases that afflict mammals (34). These diseases occur when the normal host encoded protein, PrP^C, becomes misfolded into

the abnormal, disease associated isoform, PrP^{Sc}. Prion diseases can occur spontaneously, due to mutations in the PrP sequence, or infectiously (35). This theory on the propagation of prion strains was first proposed by Dr. Stanley Prusiner as the protein-only hypothesis. Which states the theoretical model for how prions, with the same or similar amino acid sequence as normal host encoded proteins, are able too self-replicate without the presence of nucleic acids (36). The protein-only hypothesis states that a misfolded protein, prion, is able to interact with and convert a normal host encoded version of the same protein into the abnormal misfolded conformation of the prion. This newly misfolded host encoded protein has the same misfolded conformation as the original source prion and is able to misfold other normal host encoded versions of the same protein and is itself a prion. The normal host encoded protein is produced by the host and is commandeered by the prion and converted into a prion itself (37). This phenomenon can also be seen in the conversion of tau and A β to their misfolded form; they are able to seed their own conversion in a manner similar to PrP^{Sc} and are therefore thought to be prions as well as follow the protein-only hypothesis (38, 39).

Prion diseases are characterized by different and well-defined clinical diseases attributed to different PrP^{Sc} strains. It is well accepted, although not completely confirmed, that prion strain diversity lies in the different conformation that PrP^{Sc} acquires (40). Although conformational diversity is usually seen when there are different prion protein sequences (e.g., different animal species or polymorphic variants within a single animal species), it is possible that a single PrP sequence can acquire multiple different conformations. Prion strain-specific features can be appreciated at different levels,

including in pathological features of the disease, such as, incubation periods, neuropathology, clinical signs and symptoms, rate of decline, and infectivity titers among others (41). They can also be observed in biochemical features of the prion agent, such as, protease resistance, structural stability, and electrophoretic mobility (42). Considering the above mentioned similarities between prions and other proteins associated with PMDs, the concept of protein misfolded strains has been studied for tau, A β , α -synuclein and other proteins.

Cross-Seeding Hypothesis

This research focuses on the cross-seeding hypothesis in relation to the two main proteins associated with AD as the pathway for AD progression. The homologous seeding hypothesis, similar to the protein-only hypothesis, examines the mechanism for which a seed is able to act as a template in the assembly of soluble monomers. There will be decrease in the time for a misfolded protein to reach saturation in a brain, compared to an un-seeded brain (43). This process leads to aggregate formation, which can be characterized by their β -sheet structure and insolubility (44). The cross-seeding hypothesis arises from this protocol. The difference with cross-seeding relates to the seed being from a heterozygous protein versus a homozygous. Here, the nucleation period is also theorized to be decreased, but not in a time frame consistent with the homozygous seed (45). This process has been theorized to be occurring in AD. In this situation, A β will misfold in the brain of a patient and then act as a seed to induce misfolding of tau. This interaction would eventually lead to an AD clinical diagnosis (46).

Evidence suggesting that tauopathies are due to misfolded conformational variants.

Tau is known to self-propagate into distinct strains, these diseases are classified as tauopathies. Tauopathies are pathological aggregations of tau protein in neurofibrillary or gliofibrillary tangles in the human brain (47). The different tauopathies present as different, distinct conformations or strains that are able to self-propagate. Currently, over 25 tauopathies have been described (48). In addition to AD, relevant tauopathies include chronic traumatic encephalopathy (CTE), frontotemporal dementia (FTD), Pick disease (PiD), and progressive supranuclear palsy (PSD). These diseases all show signs of tau tangles in the patient brain (49). However, they all present differently clinically as well as in the pathology of the tau tangles (50). These strains of tau disease are self-propagating like prion strains (51). In a study by *Clavaguera et al.* they saw that by homogenizing the brains of patients with various tauopathies and then inoculating them into transgenic mice, the pathology of the tau tangles that developed in the mice was consistent with what was seen in the humans (52, 53).

With this, tau can be classified as a prion. Each tauopathy is different from one another based on their distinct neuropathological signatures. These differences consist of variances in the conformation of the aggregates, the area of the brain they affect, and the shape and size of their lesions. There are six isoforms of tau known in the human brain that are present and act differently in the various tauopathies (54).

Brain A β deposition is a distinctive feature of AD and it has attributed as an early change triggering accumulation, brain inflammation, synaptic loss and neuronal death (55). A β aggregates in AD brains can be found in a variety of arrangements. These different morphological deposits are reminiscent of tau inclusions in different tauopathies, or PrPSc

deposits induced by different prion strains (56). Considering this, different A β arrangements may be composed of misfolded protein that is different in its conformations. In turn, these different pathological entities may contribute to the divergent disease phenotypes observed across AD patients. Several reports have demonstrated that different conformations of A β can self-propagate in *in vitro* and *in vivo* systems, similar to what is observed for their infectious prions counterparts (57, 58). Studies have also suggested that it is the diversity of A β strains in AD patient brains that is linked to their clinical progression and presentation of the disease (59). However, this hypothesis is not proven and excludes the importance of tau in AD.

With the known variability for AD presentation both clinically and pathologically and the failure of the amyloid cascade hypothesis it could be time to start exploring other hypothesis for the progression of AD. Looking at AD and its two main proteins, A β and tau as prion-like in their behavior, mechanisms like the cross-seeding hypothesis can be used to examine the disease. This thesis will examine the possibility of A β cross-seeding tau in a strain specific manner. Through cross-seeding, the tau will misfold and aggregate in a manner that is dependent upon which strain of the A β seed it interacted with. This could possibly explain the variability in AD and clarify more of the complexities of the disease.

Materials and Methods

Protein Purification:

Streak pET28-tau2N4RCF on a LB-agar 30g/L kanamycin plate (Sigma-Aldrich). Leave the plate to incubate overnight at 37°C. Prepare culture media – distilled H₂O (dH₂O), Terrific Broth (Thermo Fisher Scientific), and 50% glycerol (Thermo Fisher Scientific). Prepare three 50 mL flasks of media and five 500 mL flasks. Autoclave media, before use. Add antibiotic to media in 50 mL flasks, kanamycin (Fisher BioReagents), and colonies from the plate. Culture media overnight at 37°C and 200 rpm. Add antibiotic, kanamycin, to 500 mL flasks. Combine all media from the three 50 mL flasks. Add 25 mL from pooled media into each 500 mL flask. Incubate 500 mL flask for 1.5 hours at 37°C and 200 rpm. Add 1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) (Thermo Fisher Scientific) to flasks to induce misfolding of protein. Incubate for 6 hours at the same settings as before. Empty flasks and equalize weight between four centrifuge bottles (Eppendorf). Centrifuge at 3,000 g for 30 minutes at 4°C. Discard supernatant and collect pellet into two 50 mL falcon tubes (Fisher Scientific). Ensure the collection of complete pellets using 40 mL STE buffer (10 mM Tris-Cl (Fisher BioReagents) pH 7.5, 100 mM NaCl (Sigma), 1 mM EDTA (Promega)). Equalize the weight of the falcon tubes and centrifuge at 5,000 g for 15 minutes at 4°C. Record weight of pellet and discard supernatant. Store pellet at -20°C.

Begin thawing pellet and resuspend in lysis buffer (20 mM PIPES (Sigma) pH 6.5, 500 mM NaCl, 1 mM EDTA protease inhibitor cocktail complete Roche (Sigma-Aldrich)). Sonicate homogenate for 5 minutes (30 pulses, 10 seconds on, 10 seconds off, 60%

intensity, output level 6, elapsed time 10 minutes). Heat lysate in two, clean, falcon tubes at 95°C for 20 minutes, mixing every 5 minutes. Transfer tubes to ice. Centrifuge tubes at 15,000 g for 20 minutes at 4°C. Transfer the supernatant to clean falcon tubes and discard pellet. Centrifuge supernatant at 15,000 g for 20 minutes at 4°C. Pool all supernatant into a glass beaker and discard pellet. Add 55% weight per volume ammonium sulfate (Fisher BioReagents) to the pooled supernatant, and incubate with a stirrer bar for 1 hour at room temperature (15-25°C). Transfer substrate to two clean falcon tubes and centrifuge at 15,000 g for 15 minutes at room temperature. Discard the supernatant and store pellet at -80°C.

Thaw and resuspend pellet in Nanopure H₂O. Begin cation exchange chromatography with AKTA machine. Wash tube on conductivity machine with 1 mL Nanopure H₂O (1 mL/min) until conductivity reaches 0. Wash with buffer A (20 mM PIPES, 50 mM NaCl, pH 6.5) and record conductivity. Again, wash with Nanopure H₂O. Using 500 µL of sample, measure conductivity to ensure it is at or below the conductivity of buffer A. Wash tube again with Nanopure H₂O. Add 0.1 mM PMSF (Phenylmethylsulfonyl Fluoride) (MilliporeSigma) to sample.

Begin loading HiTrap SP PH cation exchange column (Thermo Fisher Scientific). Clean the column with buffer B (20 mM PIPES, 1,000 mM NaCl, pH 6.5), then run a cleaning with buffer A. Wash tubing, while bypassing the column, with buffer B. After, use buffer A to reach the conductivity of 2 mL/minute for approximately 2 minutes. Ensure system and sample pump do not have bubbles. Wash the sample pump tubing with Nanopure H₂O then buffer A. After these washes running protein through tubing.

Before running the sample through the column, wash it again with buffer A for 10 minutes. Then, run the protein through the column at 0.75 mL/minute for approximately 19 hours. Pass buffer A through the sample pump, and the column to ensure all sample passes into the column.

Begin elution using a salt gradient. Assemble fraction collector with 2 mL LoBind Eppendorf tubes (Eppendorf). Collect 2 mL fractions. Analyze protein content with an SDS-PAGE Blue-Coomassie (Bio-Rad) staining. From here, pool the fractions with the highest tau content and keep on ice at 4°C.

Run dialysis on pooled protein. Dialysis overnight at a 1:100 ratio of sample to dialysis buffer (10 mM HEPES, 100 mM NaCl, pH 7.4). Run dialysis in a 10 kDa MWCO dialysis bag (Thermo Fisher Scientific) at 4°C with a stirrer bar.

Transfer protein to a 50 mL falcon tube, on ice. Centrifuge sample at 15,000 g for 15 minutes at 4°C. Move supernatant to a new tube, on ice, and discard pellet. Check concentration using a BCA assay (Thermo Scientific Pierce). To concentrate the protein, use a 10 kDa MWCO (molecular weight cut off) tubes (Millipore). To dilute the protein, use dialysis buffer. Concentration of tau should be between 6 and 7 mg/mL.

Wash filters of two 100 kDa MWCO tubes (Millipore) with 10 mL Nanopure H₂O by centrifuging at 3,000 g for 20 seconds at 4°C. Discard water from tubes. Load protein into filtration tubes, and balance weight. Centrifuge at 3,000 g for 20 minutes at 4°C twice. Clean 0.22 µm filter with Nanopure H₂O. Then run the protein through that filter and into a new falcon tube. All filters should be kept at -20°C prior to use to ensure they are cold. Aliquot the protein for later use and snap freeze them in liquid nitrogen.

Protein is stored at -80°C. Final concentration of protein can be checked with another BCA assay (60).

BCA Assay:

The assay was run at 10% dilution for the protein. It was performed in a clear 96 – well plate. Once the reagents were all added, the plate was incubated at 37°C for 30 minutes. The plate was read in a spectrophotometer at 562 absorbance.

Fibrilization of monomers:

The fibrils were made in 0.6 mL tubes (Costar). 50 uM of tau monomers were added with 10 mM HEPES buffer (pH 7.4), 100 mM NaCl, and 25 uM Heparin sodium salt (Sigma-Aldrich). The tubes were incubated in the thermomixer for 5 days at 37C and 500 rpm. After 5 days, the fibrils were analyzed via thioflavinT (Sigma) in the spectrophotometer.

Seed Preparation:

All seeds for the cells were sonicated for 5 minutes (30 pulses, 10 seconds on, 10 seconds off, 60% intensity, output level 6, elapsed time 10 minutes).

Aggregation Assays:

All reagents were combined in a black, flat bottom 96-well plate (Costar). Tau buffer (10 mM HEPES buffer (pH 7.4), 100 mM NaCl), Heparin (25 µM), and recombinant Tau 2N4R (100 µM) in HEPES (10 mM, pH 7.4) and NaCl (100 mM) were combined before the seeds were added. All seeds were at a stock concentration of 1

mg/mL. 50 μ L of the seeds were added at a 10^{-3} dilution. The reaction was incubated in a thermomixer at 25°C and 300 rpm. Reaction was imaged via spectrophotometer every 12 hours for 200 hours. Data was arranged with GraphPad Prism using an XY column graph.

The protocol for our aggregation assays was adapted to best fit the readings for this experiment. The trials were run in opaque 96-wells plates, so that they could be imaged in the spectrophotometer and preserve the volume of the reagents in each well throughout the time points. We also added ThioflavinT to each well so that the reactions could be imaged via fluorescence. The buffer contained NaCl to help decrease the amount of evaporation and HEPES to promote the aggregation of the monomers. (Figure 3)

Cell Maintenance:

Prepare medium for cells: ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) (4 mM L-glutamine, 4,500 mg/mL glucose, 1 mM sodium pyruvate, 1,500 mg/mL sodium bicarbonate) (Sigma). To DMEM add 1% L-alanyl-L-glutamine (Gibco), 10% Fetal Bovine Serum (Thermo Fisher Scientific), and 1% Antibiotic-Antimycotic (Gibco).

Prepare 100 mm cell culture dish (Denville Scientific Inc.) for cell growth: 10 mL of poly-L-lysine solution (Sigma) was added to each dish and placed in the incubator at 37C and 5% CO₂ for 10 minutes. The poly-L-lysine solution was then removed and the dishes were left to dry for 2 hours.

Cells came from Marc Diamond lab and were stored in liquid nitrogen. Vials with cells were thawed and content was transferred to a 15 mL falcon tube with 9 mL of medium. The falcon tube was then spun in a centrifuge at 200 g for 5 minutes at room temperature. Supernatant was removed and the cells were then resuspended in 1 mL of medium for each plate being inoculated. Resuspended cells were added to a cell culture dish with 10 mL of medium. Cells were grown out for 24 - 48 hours before being passaged for use in the experiments.

Cell Counting:

The cells were counted following their centrifugation and resuspension. 10 mL of the suspended cells and 10 mL of trypan blue (Sigma) were mixed. 10 mL of this mixture was added to a cell counter slide (Bio-Rad) and placed in the cell counter machine (Bio-Rad). The machine read out the total number of cells present.

Cell Trials:

12 mm diameter, round glass coverslips (Chemglass Life Sciences) were prepared with poly-L-lysine using the same method as noted for the cell culture dishes. Once the coverslips had dried, they were added to 24-well plates (Denville Scientific Inc.). The media was vacuumed from the cell culture dishes that the cells had grown on and treated with 2 mL of trypsin 0.25% (MilliporeSigma) in order to strip the cells. The dishes with the trypsin were incubated for 20 minutes at the same settings as listed previously. 4 mL of media was added to dishes with the trypsin to help with the removal and collection of the cells into falcon tubes. The tubes were centrifuged at 200 g for 5 minutes at room temperature. The supernatant was removed and the pellet was suspended. 130,000 cells

were added to each well. The cells were allowed to grow to 60% confluency over 48 hours.

Next, the cells were seeded with either PBS (Phosphate Buffer Saline) 1X (Cytiva), tau monomers, tau fibrils, 2F or 3F A β fibrils. The stock concentrations were 25 nM, for the tau monomers and fibrils, 2F and 3F. Each of the conditions were performed in triplicate. All seeds for the cells were sonicated for 5 minutes (30 pulses, 10 seconds on, 10 seconds off, 60% intensity, output level 6, elapsed time 10 minutes). For each well 1 μ L of fibrils, they were incubated with 48 μ L of Opti-MEM 1X (Gibco) and 1 μ L of Lipofectamine 2000 (Invitrogen) for 20 minutes. The wells were seeded with their respective inoculums and then live imaged at 10X at 0 hours and again every 12 hours until 96 hours. For the cells that were subjected to chronic exposure of seed, the media was not changed. However, for the cells that were exposed to acute exposure, the media was changed 6 hours after adding the seeds.

Cell Fixation:

Media is removed from the cells and wells are washed with 300 μ L PBS 1X for 5 minutes. Once the PBS was vacuumed from the wells, the cells are fixed to the coverslips with 1 mL of 10% Neutral Buffered Formalin (Thermo Fisher Scientific) and left to attach for 30 minutes at room temperature. The Formalin was then removed from the wells and the cells are washed 3 time with PBS in the same way as described before.

Cell Staining:

The last wash of PBS was removed and 300 μ L of goat serum (Sigma -Aldrich) is added to each well at 5% for 1 hour. The goat serum is diluted in Triton PBS. From here,

the goat serum was removed from the wells and the 4G8 (Thermo Fisher Scientific) was added to each well at a 1 to 1,000 dilution and left overnight to incubate on a shaker. The 4G8 dilution was also prepared in Triton PBS. The next day, the 4G8 was removed from each well and the wells were all washed with PBS 3 times, using the same protocol as described above. Goat anti-Mouse IgG Antibody (Vector Laboratories) was then added to each well at a 1 to 500 dilution, also prepared in Triton PBS. This was left to incubate for 2 hours. The wells were then washed again with PBS 3 times. DAPI stain (Millipore Sigma) was then added to each well at a 1 to 10,000 dilution and incubated for 10 minutes. The cells were washed with PBS 1X 3 times to remove any excess stain. The coverslips with the stained cells were then attached to microscope slides (Thermo Fisher Scientific) with FluorSave Reagent (Millipore). They were left at room temperature for 24 hours to dry and then transferred to a 4°C fridge for storage.

Cell Imaging:

The cells were imaged at 20X using the Lecia Thunder Imager. Two representative images were taken for each well. The images were taken at a maintained level of intensity and fluorescence.

Cell Quantification:

The cells were quantified using the ImageJ software. The images were split into channels of the different fluorescence and each channel was counted separately. The number of inoculums produced at the final time point was measured for each condition. This was done with a code developed in the ImageJ software based on their cell counter programming. The data was inserted into Prism in order to run statistical analysis.

Results.

Protein Purification from Bacteria

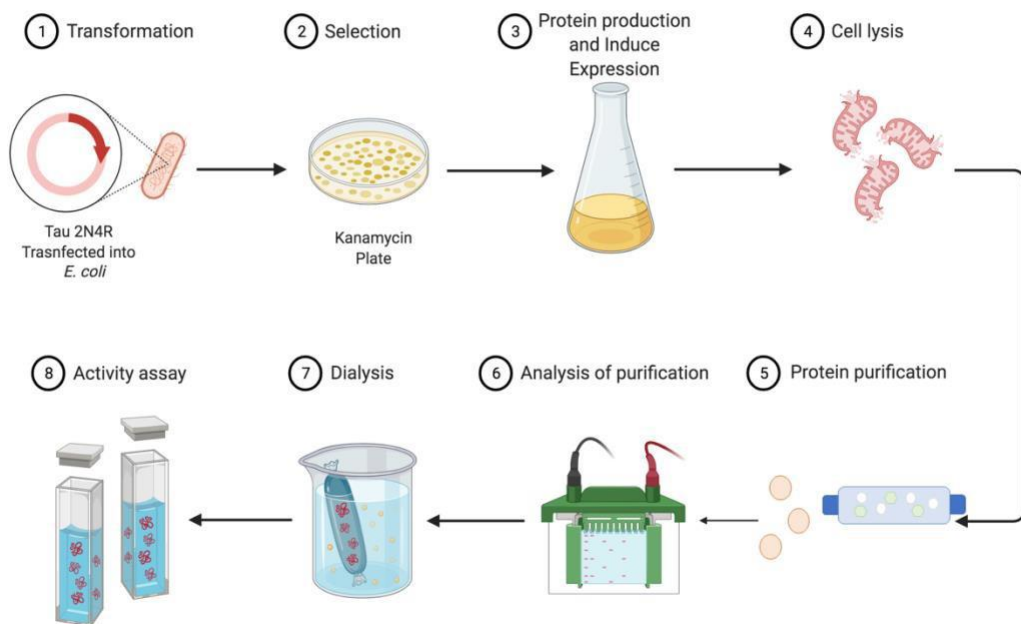


Figure 1. Schematic representation of tau protein purification. 1. *E. coli* was transfected with a plasmid expressing the 2N4R version of the human tau protein. 2. The transfected bacteria were cultured on a petri dish supplemented with kanamycin and allowed to propagate over time. 3. Colonies from the petri dish were selected and individually cultured in growing media over time. The cultured bacteria were induced to express tau protein in its monomeric form with ITPG (Isopropyl β -d-1-thiogalactopyranoside). 4. The culture was then lysed in order to isolate the protein. 5. The lysed bacteria was then purified through the ionic chambers of an AKTA machine. From here, fractions were collected containing the tau 2N4R. 6. These fractions were pooled and run through a gel in order to determine their molecular weight and purity. 7. The pooled sample then underwent dialysis to adjust the pH level. 8. The sample was then tested to determine the concentration of tau monomer that was purified. (60)

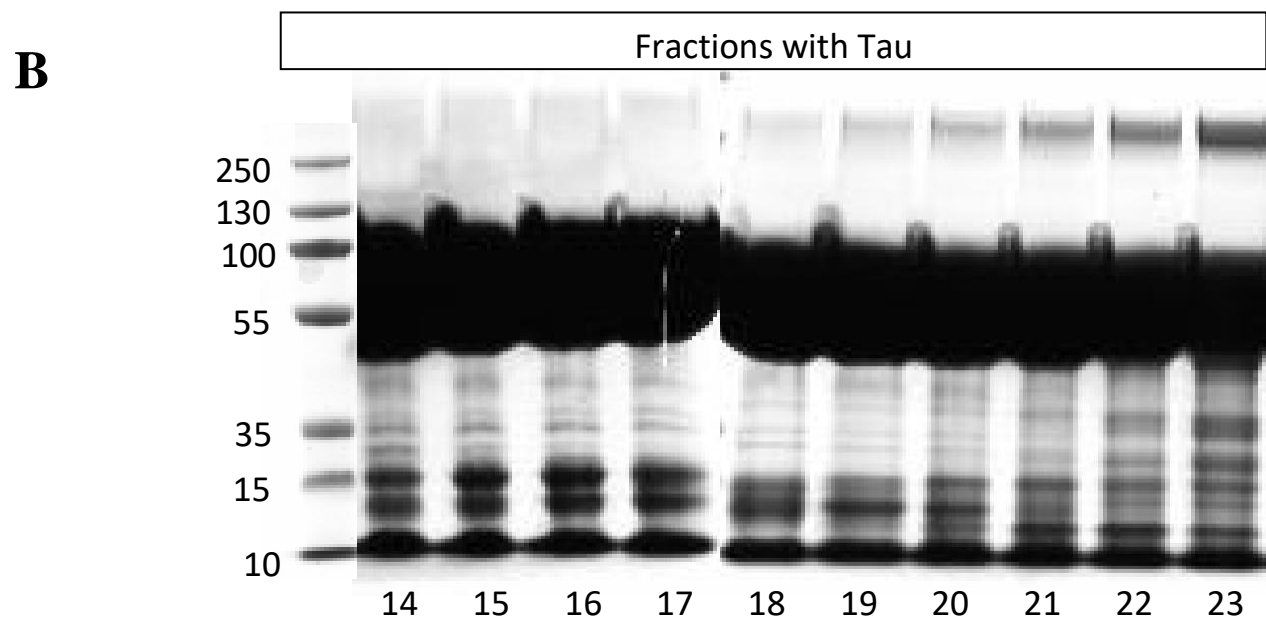
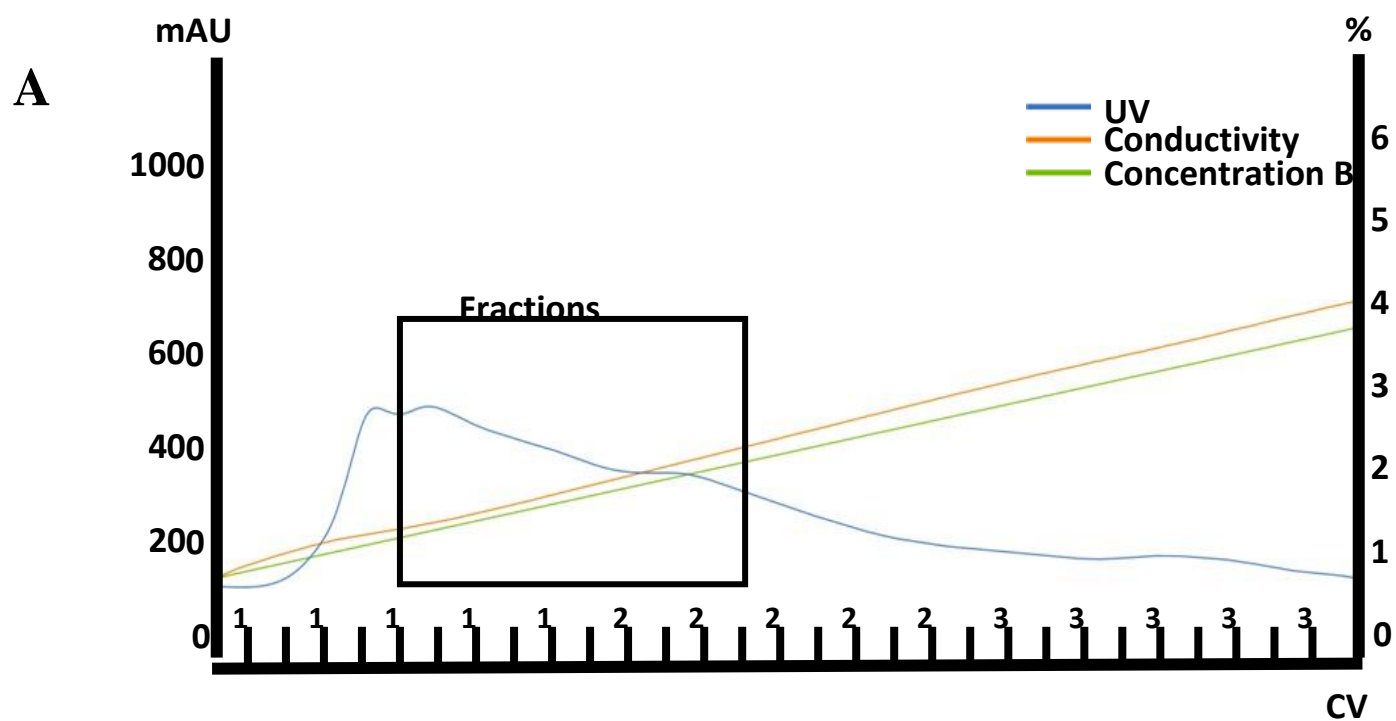


Figure 2. Results of tau protein purification. A. Fractions that were chosen to be collected from the AKTA. The fractions that displayed an increase in UV, indicating protein was moving through the column and were paired with an increase in conductivity could be isolated for use. These indications form a window, in which the tau protein was present in the buffer moving out of the AKTA and fractionalized tubes. B. Gel analysis to check the molecular weight of the fractions to ensure that tau is present in its expected molecular weight and purity.

Schematic of aggregation assays

Aggregation assays were performed in order to model the potential cross-seeding of A β strains and tau. This method allows for the interaction of the proteins without and other factors possibly altering the results. The assays were performed with tau monomer and different seeds of fibrils. This allows the fibrils to induce aggregation of the monomers within the wells and the rate of the interaction to be tracked. By adding fluorescence to the wells, we were able to take measurements of the aggregations at intermittent time points, as it was occurring. The protocol for the assays was used previously for seeding and then optimized for cross-seeding.

Optimization of parameters for aggregation assays

The protocol for the tau seeding-aggregation was first optimized for the amount of monomers needed for the optimal development of the assay. This includes the presence of a lag phase that will allow us to discriminate seeded from unseeded reactions in a dose-dependent manner, corresponding to how previous reports have described (41). For this, 10 mM, 50 mM, and 100 mM of tau monomer were run in triplicate and tested against each other to see which concentration of monomer produced the best aggregation curve. From here, 50 mM of tau monomer was chosen due to the length of the nucleation phase it produced. (Figure 4A). With this concentration, we were able to produce consistent aggregation curves with a lag phase of roughly 200 hours. This time frame was thought to be best since the un-seeded monomers would aggregate slower than the seeded monomers. Thus, allowing for a longer period for which the trials that were seeded could produce variation from one another. After this, we ran trials to determine the best settings to use on

the thermomixer. The settings from the protocol that was used previously in our lab for different proteins (A β peptides, unpublished results) were 500 rpm and 37° C. We decided to change the settings to 300 rpm and 25° C. The new settings were chosen because they reportedly cause a longer lag phase. They were also more effective at producing consistent aggregation curves and longer lag phases when I ran trials with these settings compared to those from the original protocol (data not shown). Next, trials began to test the different dilutions of tau fibrils, and how quickly they were able to induce aggregation in the tau monomer. The dilutions tested were 1×10^{-2} to 1×10^{-5} , diluted from a stock of 4.1 mg/mL. These trials were supplemented with 9 mg/mL of heparin and 4.5 mg/mL of heparin, as this reagent is known to compensate for negative charges needed for tau to misfold and aggregate in *in vitro* systems (61). When supplementing our reactions with 1×10^{-3} concentration of tau fibrils, optimal aggregation curves were obtained at both heparin concentrations. In that sense, we decided to run the trials with the most diluted concentration of heparin. (Figure 4 B) This was done to, again, maximize the nucleation phase of the aggregation curve, so differences can be spotted between the seeds. The trials were also run with no heparin, to ensure the induced seeding was not a result of the concentration of monomers in the wells.

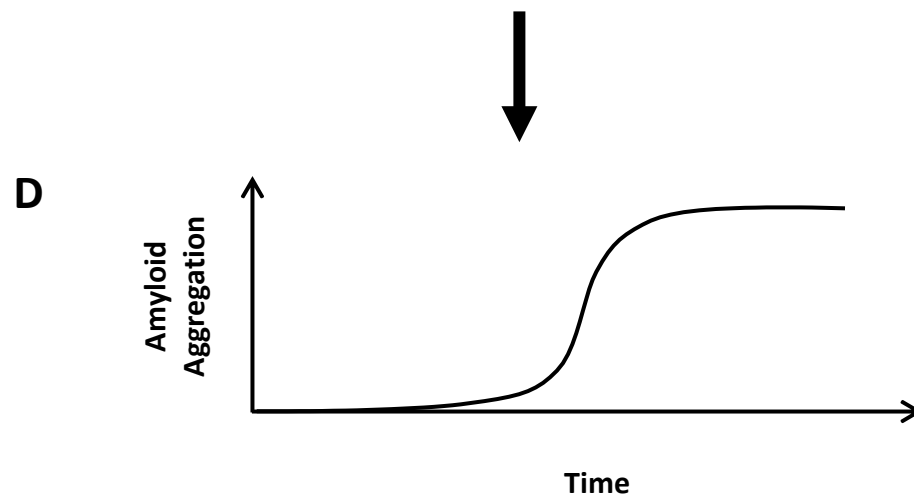
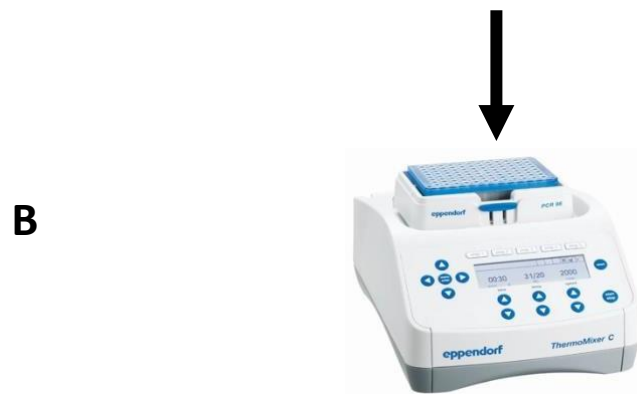
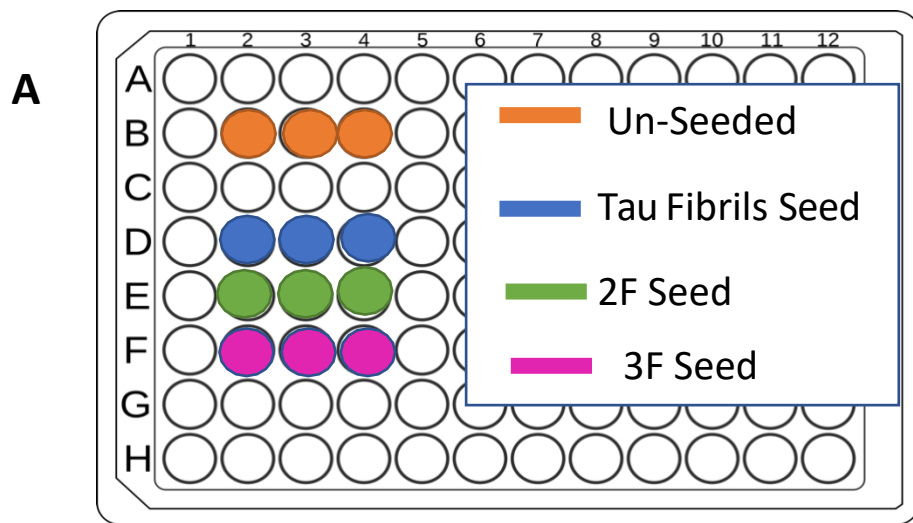


Figure 3. Schematic representation of protein aggregation assays. A. Representation of the 96-well, opaque plate and how reactions were arranged. Tau buffer, tau monomer, ThioflavinT and the various respective seeds were added to each well. B. The plates were incubated in a thermomixer at 25 degrees and 300 rpm. C. The aggregation assays were analyzed with a spectra max at approximately 12-hour intermittent time points. D. Data collected from the SpectraMax generated sigmoidal curves displaying differences in the rate of the aggregation between different seeds.

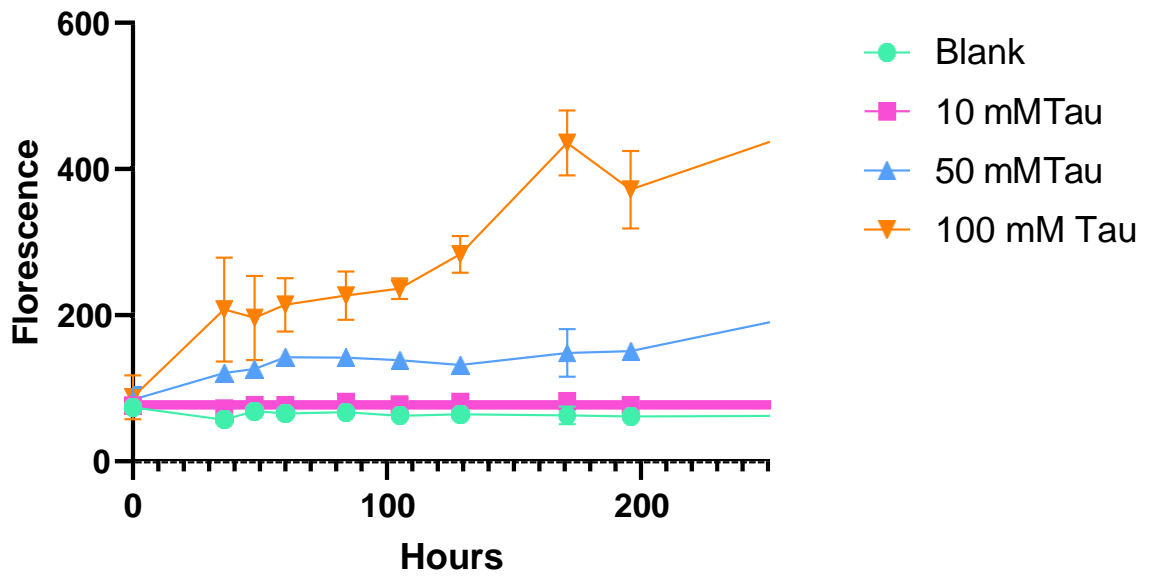
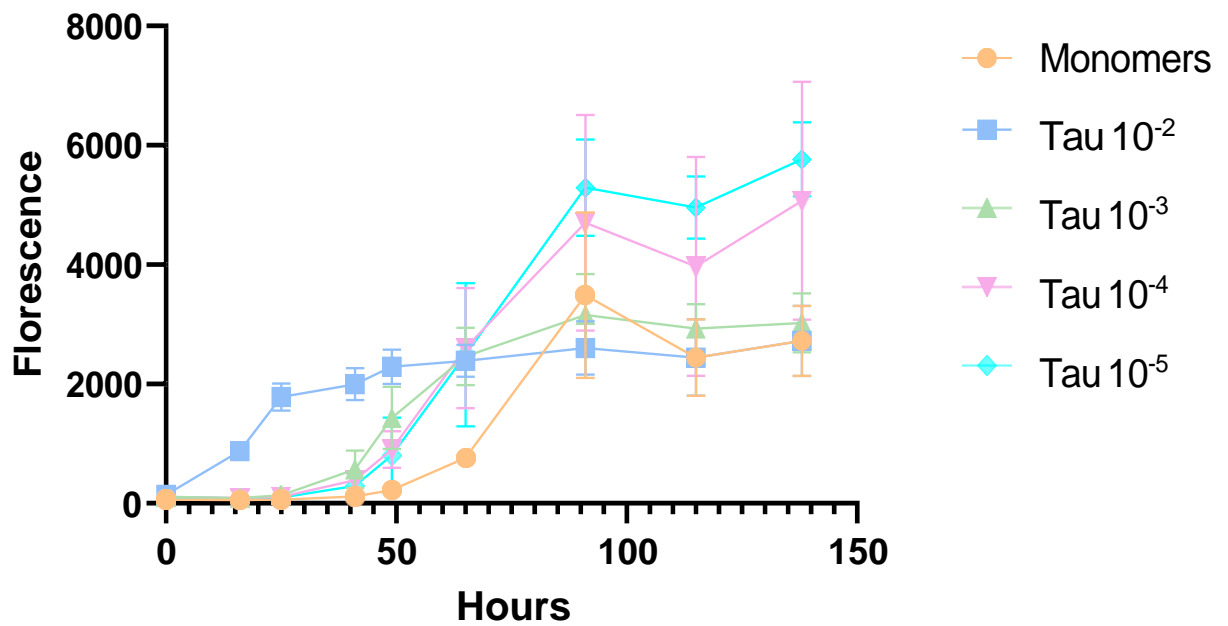
A**Tau Monomer Variations****B****Variations of TauSeed**

Figure 4. Standardization of protein aggregation assay. All conditions of the assay were optimized in order to ensure there was enough time in the nucleation phase of the aggregation curve in order to decipher possible differences in the seeds. A. The first aspect we optimized was the concentration of the tau monomer being added. We tested this with 10 mM, 50 mM, and 100 mM. The 50 mM was the lowest concentration that aggregated, and had the longest nucleation phase, therefore, it was chosen. The concentration of heparin as well as the settings used to induce the aggregation were also optimized (not shown here). B. Lastly, we tested different dilutions of tau fibrils to determine which was optimal for our positive control as a homologous seed. The fibrils that were diluted at 1×10^{-2} induced aggregation at a much faster rate than the rest of the dilutions, which were all similar in their aggregation rates. The 1×10^{-3} was chosen since the fibrils more dilute did not have a large difference.

Different conformations of A β fibrils weren't able to cross-seeds tau protein in a strain-specific manner

Our aggregation assay showed no significant differences between the 2F and 3F A β seeds. The rate of aggregation as well as the final level of fluorescence were analyzed and while there is a differences between the tau seed and the A β seeds, there is no difference in the two A β seeds. The A β seeded wells were significantly different than those that were un-seeded with just the monomer. (Figure 5 and 6)

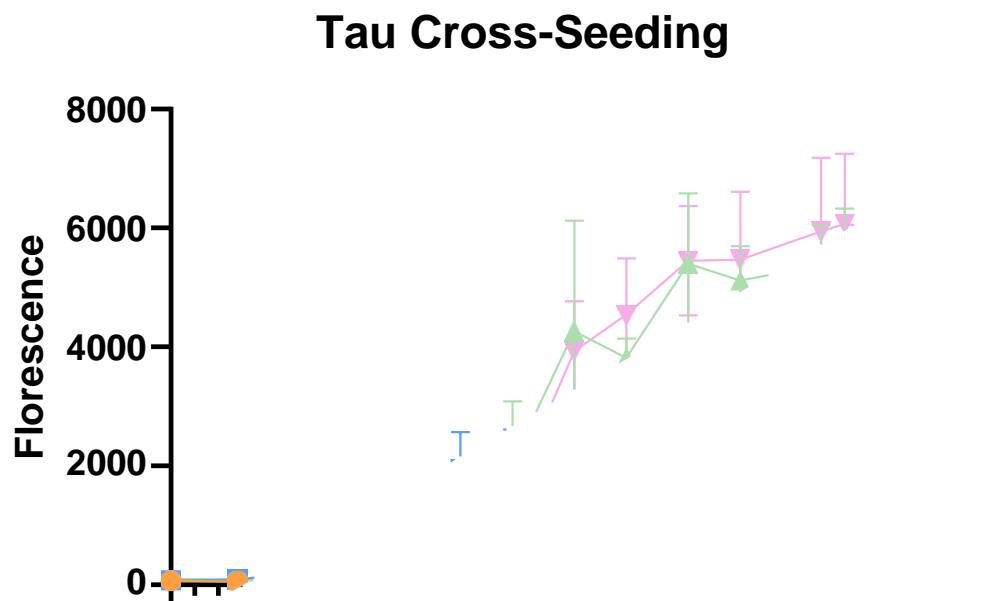
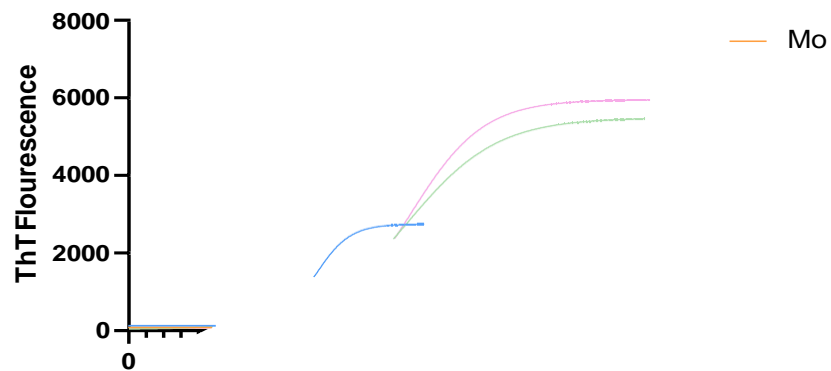
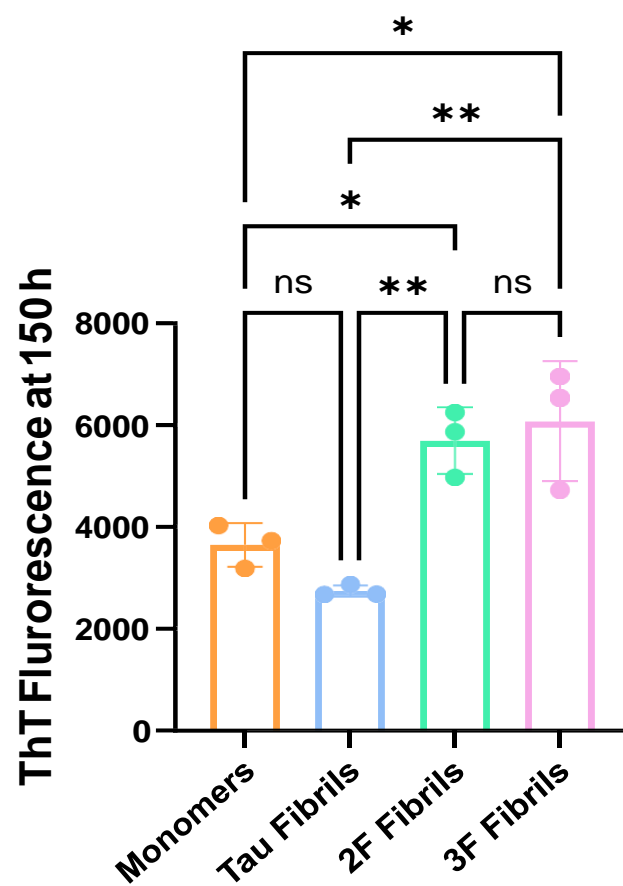


Figure 5. Aβ Cross-seeds tau in protein aggregation assay. The Aβ fibrils were able to seed the tau monomers in order to produce aggregation. The heterologous seed (2F and 3F Aβ) had a longer lag phase than the homologous seed (tau). However, it was shorter than the unseeded monomers.

A**B****C**

	2F Fibrils	3F Fibrils	Tau Fibrils	Monomers
T50	76.41	81.19	53.86	88.2

Figure 6. Statistical analysis of protein aggregation assays. The data from the aggregation assay were analyzed via Boltzmann Sigmoidal analysis. A. The curve was standardized to show the difference between the plateau values and the length of the nucleation phase.

B. The differences in the fluorescence level at the plateau phase of the curve. The differences between the monomers and the tau fibrils are not significant. This shows that the morphology of the aggregates were similar. The final fluorescence of both the 2F and 3F A β strains are significantly different from tau fibrils. However, the 2F and 3F are not significantly different from each other. C. The time to 50% of the final fluorescence at the plateau was determined for each seed. This indicates the rate of the aggregation.

Tau RD HEK cell behavior

The cells used for this project were developed and given to the lab by the Marc Diamond lab. These are a monoclonal line of HEK293 cells that express tau RD (repeat domain). These cells are purified from rat kidney, and act as reporter cells that fluoresce with GFP. The tau that has been transduced into the cells can aggregate within them, when promoted, and this interaction will lead to inclusions that are visible via a bright green fluorescence. (62)

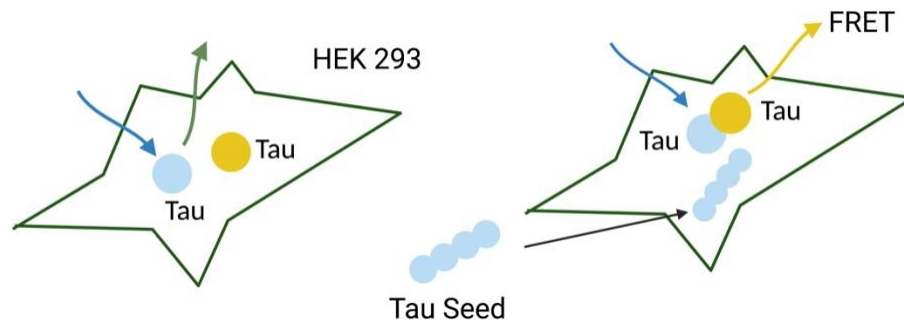
These tau biosensor cells can show various morphologies when seeded and have been shown in previous work to be susceptible to seeding when exposed to assorted tau fibrils (Figure 7). The positive control for this project is tau fibrils, since they are a heterologous seed for these cells. Tau fibrils will be able to interact with the tau that has been transduced into the cells to generate inclusions in the cells, also known as punctas. We determined the effect of the other seeds by counting the total punctas generated at the end of the trial and compared them to the number generated by the tau fibrils.

Optimization of seeding in tau cells

We began the optimization of seeding the tau cells by testing their efficacy against preformed tau fibrils considering a chronic exposure regime. For each test condition, we maintained the same seed concentration. (Figure 8) After the first trial, we decided to test if the seeding effect we were seeing was caused by the initial exposure of the seeds inducing the cells to aggregate or if it was the chronic exposure to the seed that was leading to the aggregation. The trial was run with media changes, therefore, removal of excess seeds, at 6 hours and 12 hours. The data from this trial showed that aggregates were still being

formed, even when the seeds were removed at 6 hours. (Figure 9) 6 hours was chosen as the acute seed exposure time length, since it was still able to induce seeding, but was a fairly short exposure.

A



B

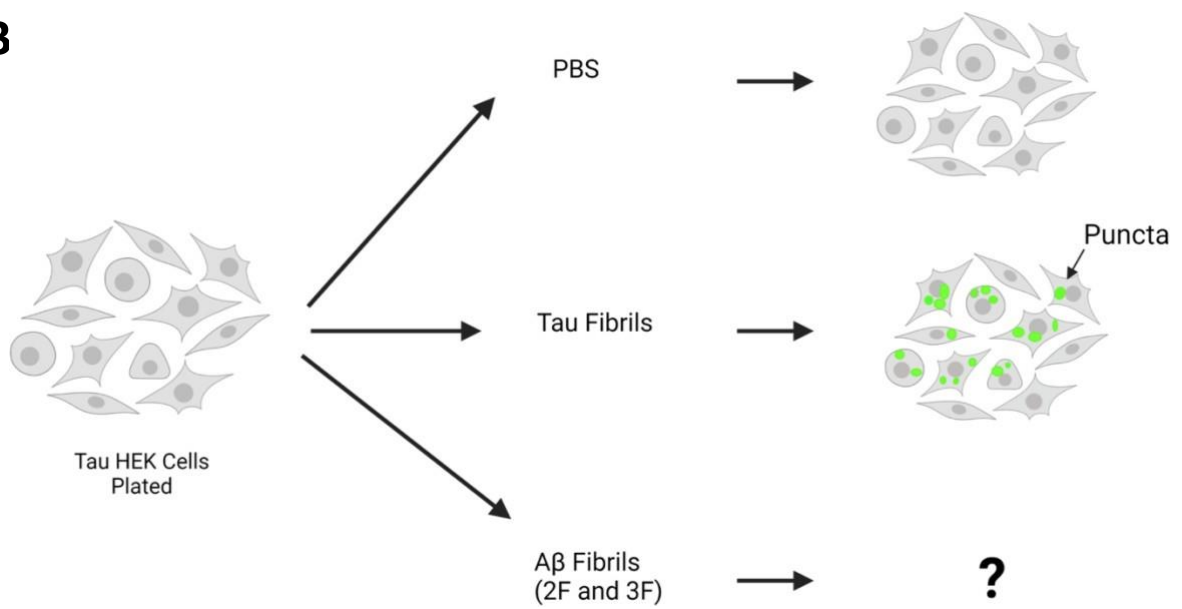


Figure 7. Schematic representation of HEK293 tau cells behavior and assays. A. The cells react via FRET-biosensor seeding assays. The cells used are HEK293 and can stably express tau RD (repeat domain), which is fused with a yellow, cyan fluorescent protein. When these cells are exposed to seeds of different protein fibrils, the tau RD in the cells can aggregate and this will produce a FRET signal. This signal is visualized as a green light with live imaging. B. In the trials in this project, the HEK cells will be plated and grown out, producing no significant fluorescence at this point. From here, they will be inoculated with the various seeds. It was expected that PBS, the negative control, would produce no aggregates. The tau fibrils, the positive control, was expected to produce a large number of punctas. This project looked to study what the effect the A β seeds would have on the cells.

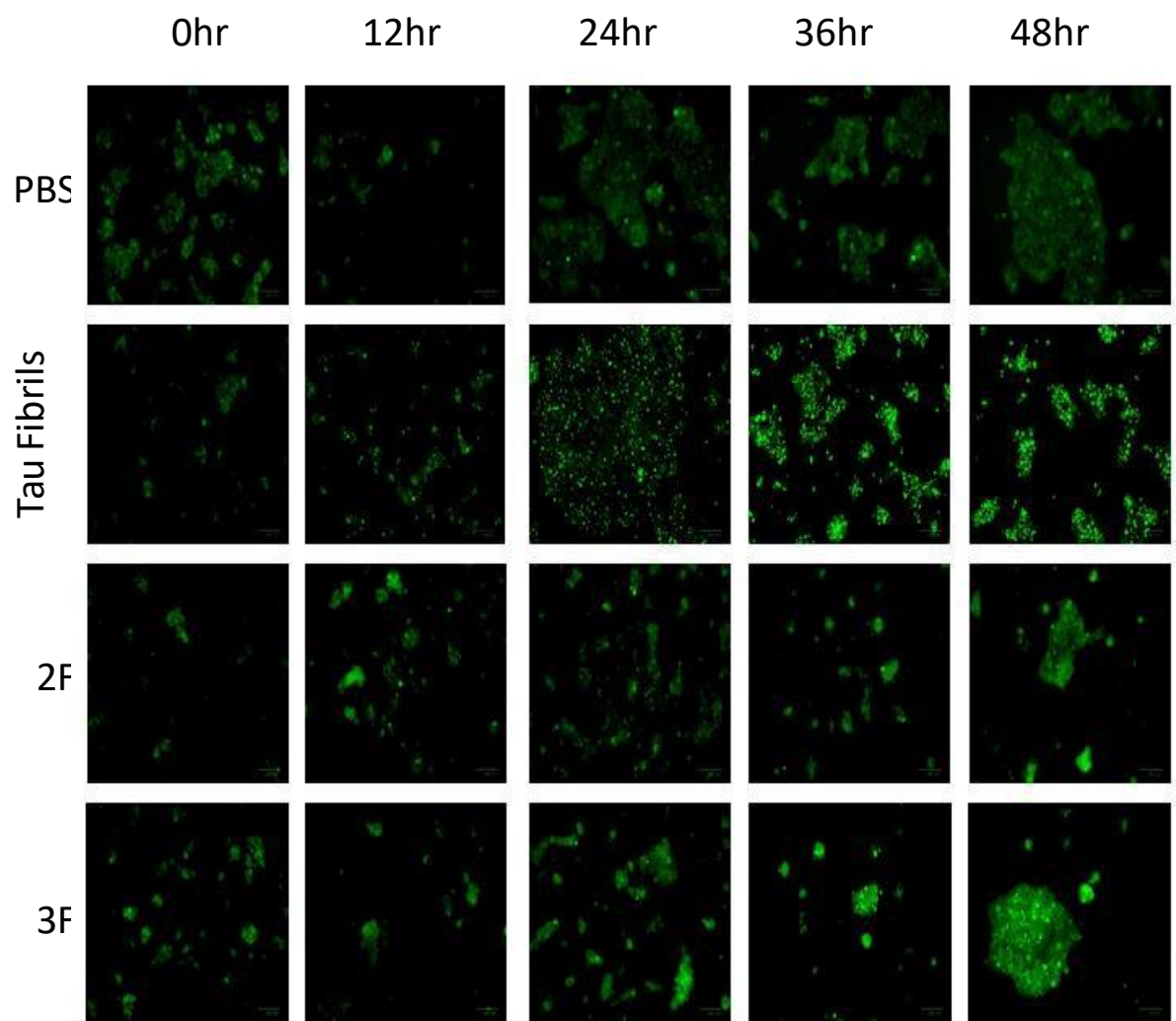


Figure 8. Standardization of heterologous seeding in cells. We began the optimization of the cell cross-seeding protocol with test the effect of the seeds when they are not removed from the cells. This trial had a continuous interaction between the seed and the cells. This step in the optimization phase was done to determine the effectiveness each type of seed had at producing aggregates in the cells. There were inclusions formed in the cells that were seeded with the tau fibrils, the 2F and the 3F.

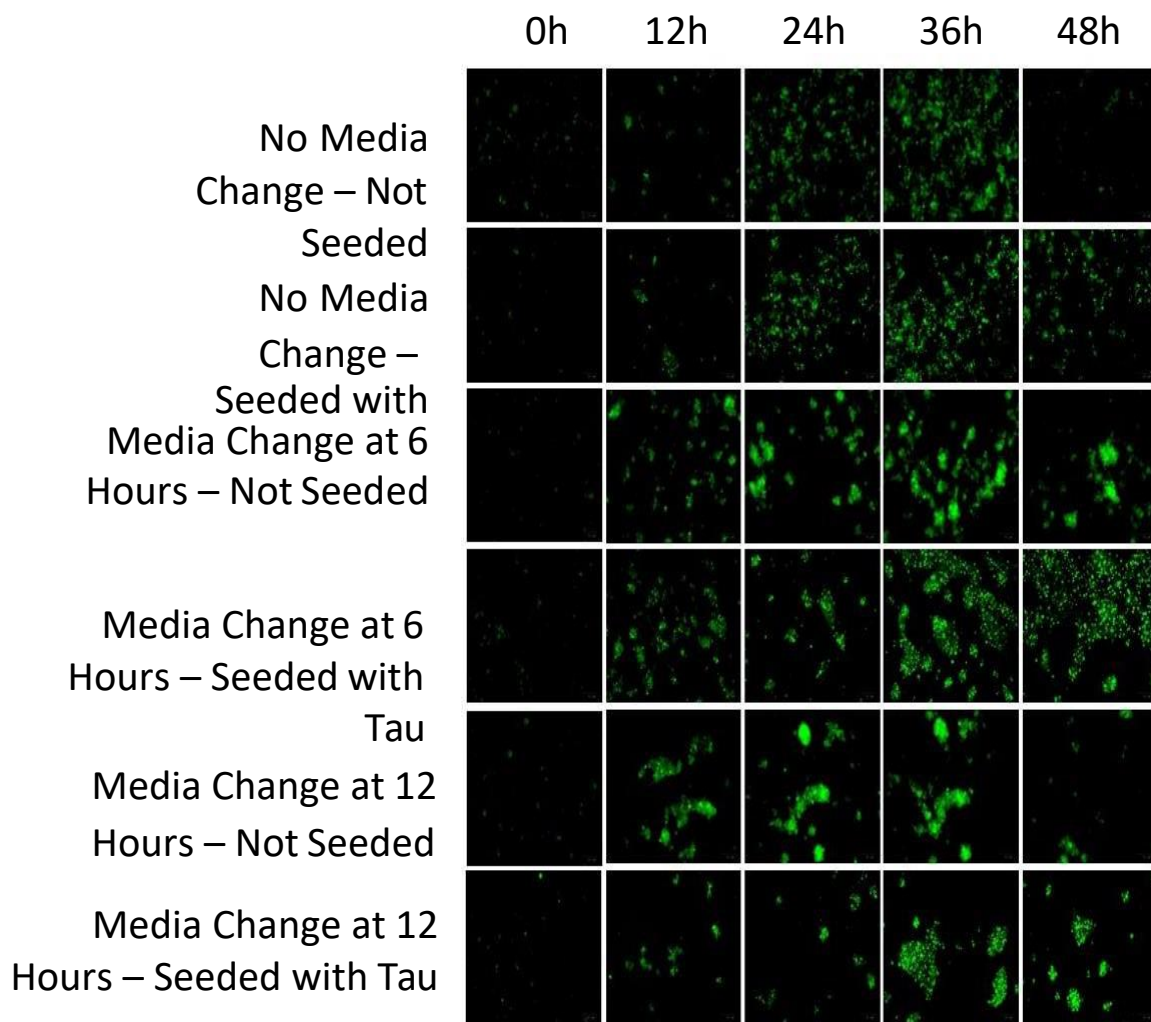


Figure 9. Standardization of acute exposure seeding in cells. The positive and negative control groups, PBS and tau fibrils, were used to determine the inoculation potential of acutely exposed cells. Puncta's were formed in all of the wells that had tau fibrils, regardless of how long they were exposed to the seed. The shortest period of time where the seeds could interact and induce aggregation was 6 hours.

A β seeds promote tau aggregation in HEK cell models in strain-specific manner

Following the protocol established by the Marc Diamond lab (42) we seeded tau RD HEK cells with A β fibrils to produce aggregates. The A β fibrils were able to cross-seed with the tau cells in order to form aggregates. These inclusions were visible via live imaging. (Figures 10 and 11). The cells were then fixed in order to be visualized at a 20 times magnification. Upon the initial visualization, and qualitative analysis of the live images, we were able to deduce a difference between the cells that had been seeded with the 2F fibrils of A β from those seeded with the 3F fibrils. It was determined that the A β strains, 2F and 3F were able to seed the tau cells in a strain specific manner. Quantitative data was formed in order to establish that the number of punctas formed in the cells varied significantly between those seeded with the different strains. This was determined using imaging and counting the inclusions in the fixed wells. All of the conditions were run in triplicate and for each well 2 representative images were taken. These 6 pictures for each condition were quantified using programming written in ImageJ. The program was able to count the number of punctas formed in each image.

Cells seeded with chronic exposure to the seeded

Chronic exposure of the cells to the seed maintained a high level of punctas produced for all of the seeded groups. In this trial, the seeds were left to interact with the cells for the full 96 hours of the trial. With this extended exposure, the cells produced a larger number of aggregates with all the seeds. (Figure 10)

Cells cross-seeded less effectively with acute exposure to the seed

When the cells had acute exposure to the A β seeds, 6 hours, they were less effective in producing aggregates compared to the chronic exposure. The positive control, tau fibrils, were still able to produce punctas as effectively when the seeds were removed from the wells. This could be implying that it is not the initial contact with the seed that is leading to the aggregate formation, and the tau cells becoming misfolding and inducing misfolding in neighboring cells. (Figure 11)

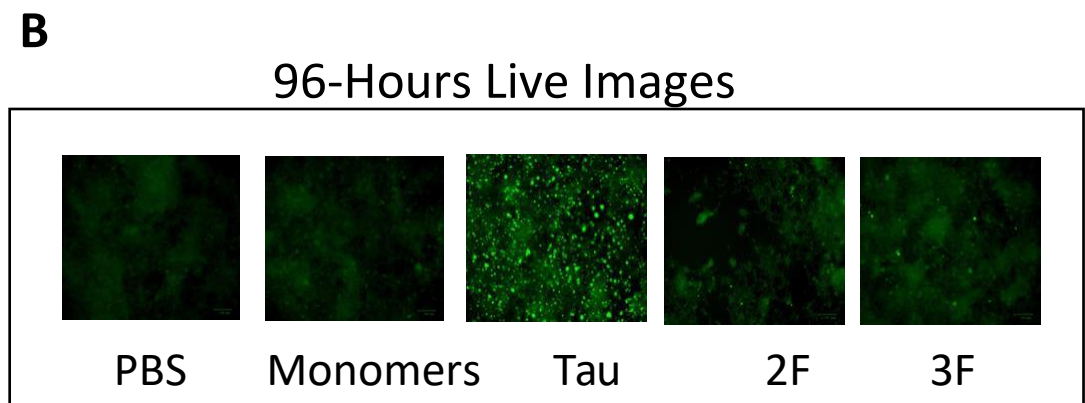
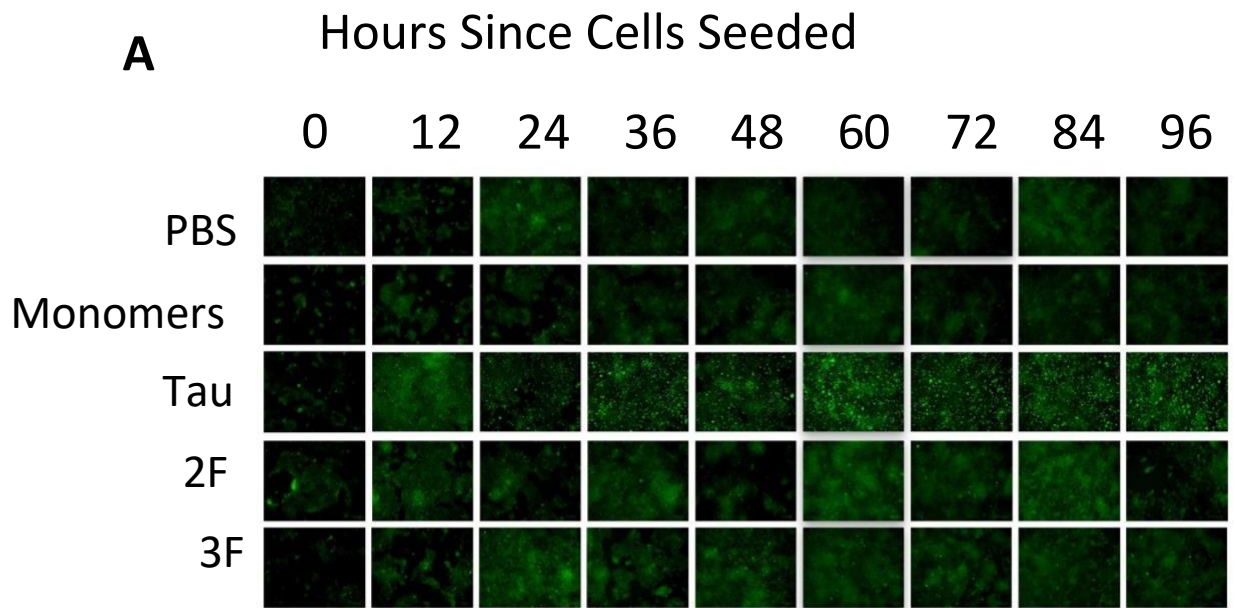


Figure 10. Live image qualitative analysis of chronic seed exposure in cells. A. Live images were taken of the cells, every 12 hours, of the 96-hour trial. All of the cells were imaged with GFP in order to visualize the aggregation over the course of the trials. We did not see aggregation in PBS or monomer-treated cells. Inclusions can first be visualized at 12 hours in the tau fibril treated cells, and at approximately 60 hours in the 2F treated cells and 48 hours in the 3F treated cells. B. Photos from each condition at 96 hours are enhanced to better visualize the inclusions that were formed. There appears to be a difference in the number of inclusions produced in the 2F versus the 3F treated cells.

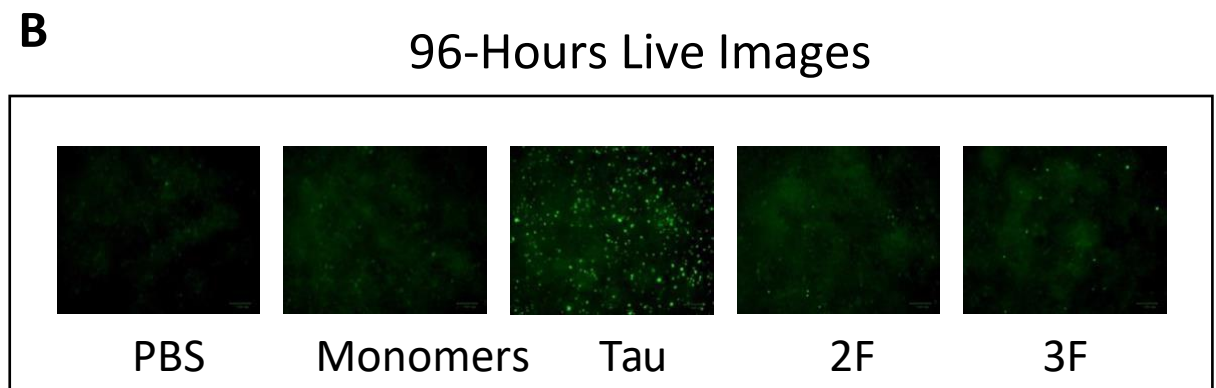
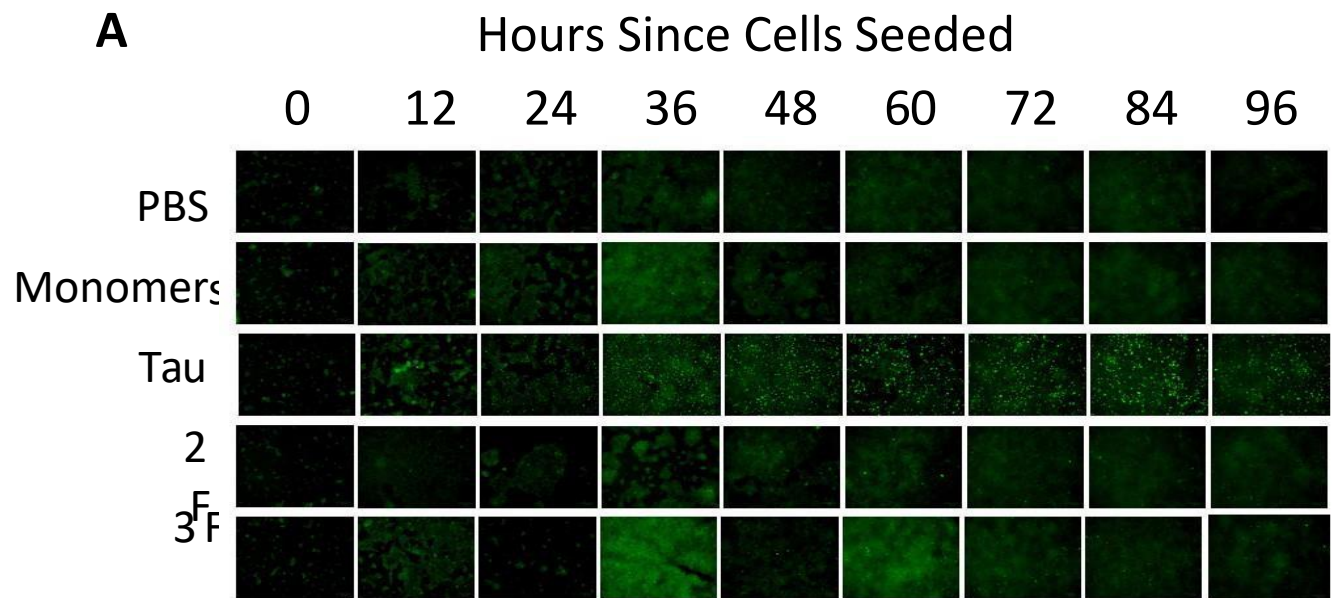
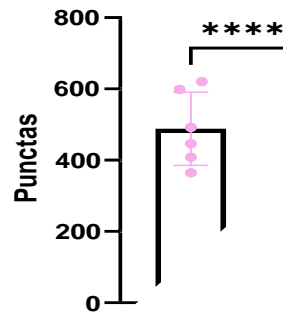
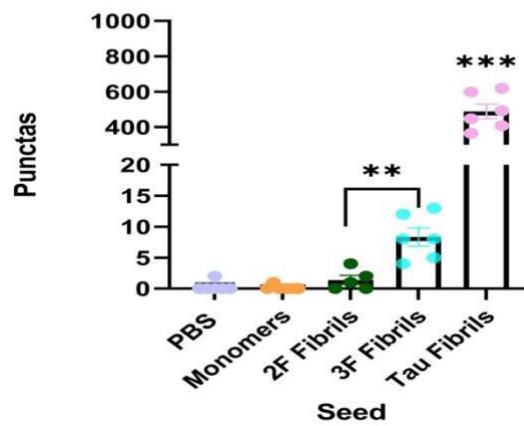


Figure 11. Live image qualitative analysis of acute seed exposure in cells. A. Live images were taken of the cells, every 12 hours, of the 96-hour trial. All of the cells were imaged with GFP in order to visualize the aggregation over the course of the trials. We did not see aggregation in PBS or monomer-treated cells. Inclusions can first be visualized at 12 hours in the tau fibril treated cells, and at approximately 60 hours in the 2F treated cells and 48 hours in the 3F treated cells. B. Photos from each condition at 96 hours are enhanced to better visualize the inclusions that were formed. There appears to be a difference in the number of inclusions produced in the 2F versus the 3F treated cells.

A Variations in Tau Seeding Efficacy

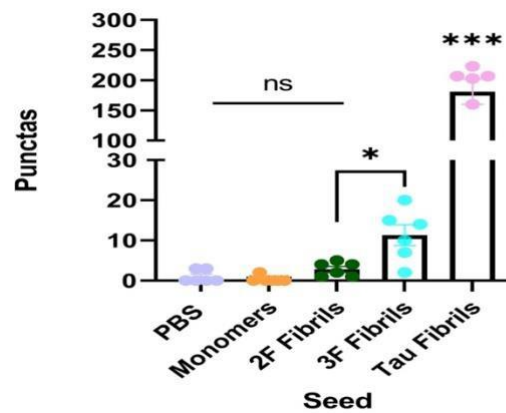


B Punctas Formed at 96 Hours



Chronic Exposure

C Punctas Formed at 96 Hours



Acute Exposure

Figure 12. Quantification of punctas produced in cross-seeding. The inclusions in the fixed cell images were quantified to determine the difference in the number of puncta's produced by each seed. A. The cells that received chronic exposure to the seed had a significant difference between the number of puncta's produced by the 2F versus the 3F. There was also a significant difference between the positive control (tau fibrils), the negative controls (monomers and PBS), and the A β strains. B. The significant difference between the number of puncta's visible in the 2F and 3F strains was still produced in the cells that experienced acute exposure to the seed. There is a significant difference in the number of puncta's between the tau fibrils and the A β strains, but not the PBS or monomers with the A β strains. The number of inclusions produced in the cells by each seed was counted using ImageJ. Each condition was run in triplicate and 2 representative images were taken from each triplicate.

Proposed mechanism for A β and tau cross-seeding in patients

Based on the data shown in this project, we hypothesize the mechanism for which A β is able to cross-seed tau, in a strain specific manner, is as follows. Initially, A β will be formed in the brain through the APP pathway. This protein will spontaneously misfold into one of the many possible different conformations. This misfolded A β will then act as a seed to induce misfolding of other A β in the brain into the same conformation, or strain, of the original A β . These proteins will then interact, either directly or indirectly, with the monomeric tau to induce tau fibrillization

in a manner related to the strain of A β that is being produced. This will lead to strain-specific pathological and clinical effects on the patient.

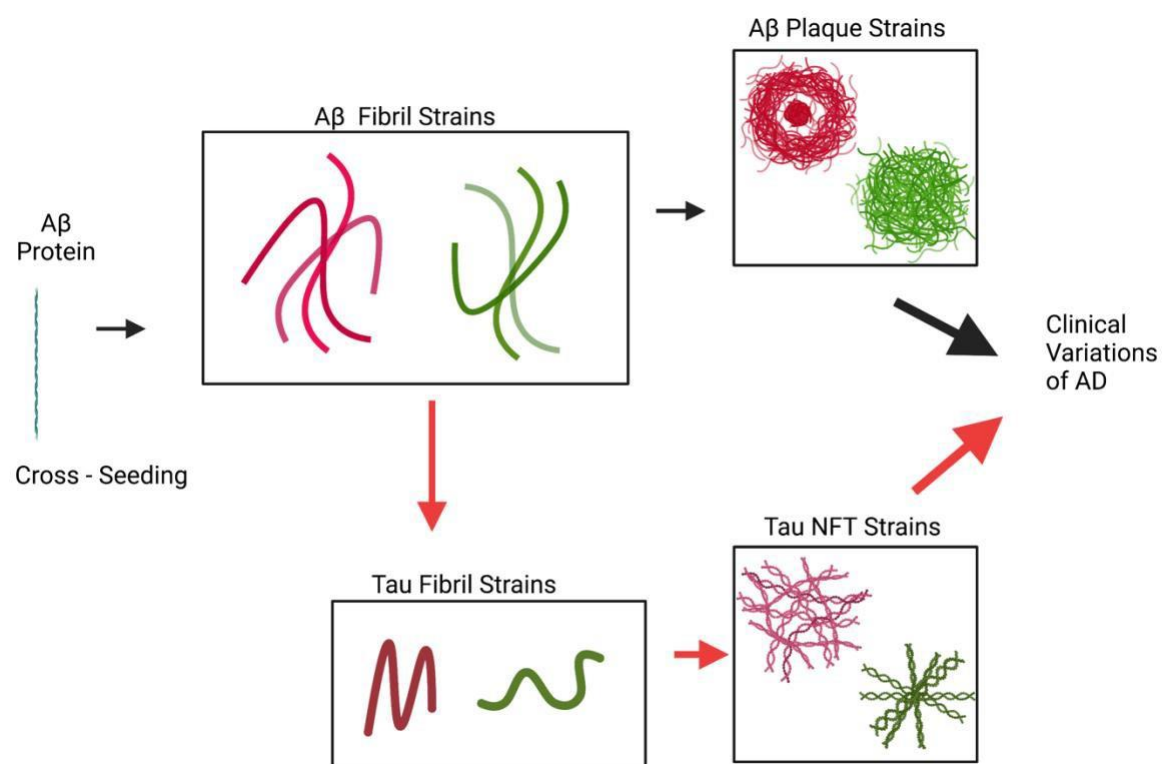


Figure 13. Proposed cross-seeding behavior of A β and tau. The proposed interaction between the tau and A β in this project begins with the APP protein producing A β protein. From here the A β protein will be misfolded into different strains, that have variation in their morphology and biochemistry. These strains will both form into plaques as well as interact with tau fibrils. The tau fibrils had been produced via the MAPT gene and their misfolding will be dependent upon which strain of A β they interact with. This will lead to NFTs that correlate with the A β plaques and eventually the clinical progression of AD in the patient's brain.

Discussion and Conclusion

Many different theories currently exist for the progression of AD. The previously favored hypothesis, the amyloid cascade hypothesis, has been ineffective as a template for developing cures for AD for the previous few decades. Due to this, there has been a rise in the “prion-like” mechanism theory for the spreading of AD in the brain. This is the basis for which the hypothesis in this project was developed. Previous literature has shown that A β and tau are self-propagating and can act in strain like manners. Examples of this being tauopathies and morphological variations of plaques in AD patients. However, this theory is not able to address the complexities of the disease and the root of the variations that cause so much difficulty in discovering a treatment for AD. As mentioned, it has been established that the physiological presentations of A β plaques and tau tangles in patient brains can vary. However, the exact mechanism for why this is occurring is still unknown. Previous literature has suggested that A β and tau could be acting in strain specific manners, like those seen in prion diseases. Prion disease are also known to self-propagate, where their misfolded form can act as a seed to a native protein. This mechanism has been suggested to be occurring in the brains of those diagnosed with AD, where misfolded A β is able to induce the misfolding of tau, therefore seeding the protein (63). The research in this paper suggests that A β is able to misfold and self-propagate in a strain specific manner and then cross-seed tau to misfold into a strain dependent upon which strain of A β it interacted with. This interaction could be the explanation for the variability in the clinical signs and progression of AD.

A β deposition in the brain is a distinctive feature of AD and it has attributed as an early change triggering tau accumulation, brain inflammation, synaptic loss and neuronal death (48). As discussed above, A β aggregates in AD brains can be found in a variety of arrangements, including intracellular aggregates, diffuse plaques, vascular deposits, soluble A β oligomers, dense-core senile plaques, as well as many others (64). These different morphological deposits are reminiscent of tau inclusions in different tauopathies or PrP^{Sc} deposits induced by different prion strains (65). Considering this, it is plausible that different A β arrangements could be composed by conformationally different misfolded A β .

The strain variation in A β and tau has previously been established. However, if there are multiple strains of each of these proteins in different AD patients, has not yet been determined. It is known that AD is a tauopathy, and that different tau morphologies produce distinctive clinical presentations when the patient brain is affected by the NFTs (66). Previous data from our lab looked into the strain variability of the 2F and 3F A β used in this project. While these synthetically produced strains were already known to show morphological and biochemical differences, they were also tested in their seeding capabilities. The data produced in our lab showed that the 2F and 3F strains could seed monomeric A β in a strain specific manner. This was shown via aggregation assay as well as through animal inoculation. Differences were seen in their rate of aggregation as well as in the behavior and brain pathology in transgenic mice (67). We tested to see if this strain variability remained when the fibrils were used as a heterologous seed, and not just as a homologous seed.

Hyperphosphorylated or misfolded tau is an important contributing factor in AD. It is widely accepted that cognitive decline correlates more directly with tau rather than amyloid pathology (68). In that sense, differences at the tau level need to be considered when trying to explain clinical variation in AD. Tau is involved in several, clinically diverse diseases grouped as tauopathies (42). Importantly, tauopathies are associated with unique tau arrangements that propagate disease-specific features in susceptible hosts (69). These pathological differences observed across tauopathies may be due of the different tau isoforms preferentially recruited in each disease (47), among other factors. More recent reports show that clinically different tauopathies are actually linked with different structures of misfolded tau (62). These experiments demonstrate the conformational plasticity of misfolded tau aggregates, and strongly suggest an active role of tau polymorphs across tauopathies.

Using the two methods described in this project we were able to study the direct interaction of A β and tau without any possible interference from other, compounding factors. This was an appropriate option for this paper considering this will be the preliminary data for *in vivo* protocols that will look to test this hypothesis in a model more similar to what is seen in patients. This also allowed for tracking of the interaction as the proteins aggregated. By doing this we can see not only the differences in the groups at the final time points of the protocols, but also in their rate of aggregation. While both of these systems are synthetic, it is a clear way to test the direct relationship between the A β and tau proteins. Future work in this hypothesis will look at *in vivo* models to test the protein-

protein interactions. This will give a more comprehensive look into the A β and tau interaction as it is possibly happening in humans.

The first step in researching our proposed mechanism began by determining if there was a difference in the cross-seeding with the two strains of A β in a protein model. It was necessary to lengthen the nucleation phase, or lag phase, of the aggregation curve so that there would be enough time to determine a difference, if one existed, between the different seeds. With optimizing the volume of the tau monomers, the settings on the thermomixer, and the dilution of the seeds, we were able to establish an easily reproducible result. The aggregation induced by the seed was confirmed by negative controls testing for the effects of the seed, the effects of the heparin, and the effects on the fluorescence by the buffer. We did this by running the trials with the final settings but no seed, no heparin, or no tau monomer. For each of these trials no aggregation was seen in the wells. This highlights the importance of each of these reagents in producing aggregation. The trials were consistently able to reproduce aggregation curves with similar nucleation phases and levels of fluorescence in each group tested.

The data from the aggregation assays revealed a difference in the rate of aggregation between a homologous seed versus a heterologous seed. The rate of aggregation is similar between the 2F and 3F fibrils, but both are different from the tau fibrils. This suggests that the seed used has an effect on the aggregation. There is also a significant difference in the final fluorescence at the plateau between the tau seed and the A β seeds. This suggest that the morphology of the aggregates being forms are different.

After the aggregation assays were optimized and run we tested this data in a cellular model. These cells had already been used in order to show seeding and have been able to produce morphologically variable aggregates dependent upon the seed they were exposed to. We used a protocol developed by the Marc Diamond lab (70) in order to seed the cells with tau fibrils. We optimized these trials before optimizing the cross-seeding. We did this by determining the settings for sonication of the fibrils as well as the volume of lipofectamine necessary for the cells to be able to interact with the fibrils. Once this was established we tested the cross-seeding of the cells with the A β fibrils.

Previous literature (70) suggested that there is a quantitative and qualitative difference in the punctas formed by the seeded cells when they experience chronic versus acute exposure. We decided to test this to see if it was happening using our optimized protocol. We decided to run the acute exposure at 6 hours, 12 hours, and 24 hours. We did this to test the minimum amount of time necessary to induce the aggregation in the cells. There was a visual qualitative difference in the preliminary data of the acute exposure when compared to the chronic exposure. We then decided to fix the cells from these trials, at every 12-hour time point, in order to stain for the nuclei and the A β . We were also able to image these fixed cells at a higher magnification, allowing for a better analysis of the number of punctas being formed in each group. When deciding how to qualitatively determine the significance between the seeding of the cells in the various groups, we decided to count the number of punctas present at the end of the trial. This was determined because each well began with approximately the same number of cells, so the differences in the number of punctas formed would be representative of the efficacy of the seed in

inducing aggregation in the cells. This analysis showed a significant difference between the number of punctas being produced between the strains of A β .

Both conditions of cell exposure, chronic and acute should a significantly larger number of punctas being formed by the tau fibrils. Interestingly, there was a significant amount more punctas formed by the end of the chronic exposure trials than the acute exposure. This indicates that the seeding effect will begin quickly upon exposure, but the longer the cells have to interact the more aggregates can form. Suggesting, while the fibrils can seed a few cells, and the tau in the cells can self-propagate and spread the aggregation to other cells, it's not as effective as the fibrils. The chronic exposure cells also had a few more punctas formed with the A β seeds than the acute exposure, but not as large of a difference as the tau fibrils. There was a significant difference between the 2F and 3F seeded cells in both the chronic and acute exposure tests. This suggests the tau was able to aggregate in a manner specific to the A β it was seeded with.

Future work, using this cell protocol, could examine the morphology of the punctas as well as the relation of the 4G8 stained A β to the inclusions. Other future work for these trials will include tests to decipher the morphology of the aggregates being formed by the cross-seeding. Biochemical tests could be used in order to determine rather the aggregates being formed in the cell model are variable between the groups being tested. Also, future research could explore the different morphologies that A β could be misfolding into within a human brain, and how many morphologies exist. Beyond that, further research could explore the number of strains of tau that exist across AD patients.

The optimization of a cross-seeding aggregation assay and cross-seeding analysis on HEK cells will prove to be valuable when looking at the other proteins associated with AD. Also, this data is a first step toward developing a possible new hypothesis for which AD can be studied, both for a cure as well as prevention. The data from this project suggests that there can be a link between the A β strain, thus plaque formation, and subsequent tau strain, therefore tangle formation. Since the morphologies of both of these are known to affect the neuronal toxicity and connections in the brain, this could be a key feature in why there is diversity in clinical signs and progression of AD.

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