STANDARDIZING THE SYNUCLEIN PFF MODEL FOR PARKINSONS DISEASE

PART 1: CHARACTERIZING THE INJECTATE

MJFF/ASAP WORKSHOP - JANUARY 27, 2022

WORKSHOP GOALS

The alpha-synuclein PFF model is a widely used model of Parkinson's disease. However, there are several technical and biological considerations that must be acknowledged when using the alpha-synuclein PFF protein to develop an in vivo model of PD as these can greatly impact the outcomes observed.

The goal of this workshop is to

1. Discuss biophysical and biological characterization of the aSyn PFF protein to improve understanding of injectate and resulting pathology.
2. Recommend minimal QC and characterization of the aSyn protein for labs using the aSyn PFF model.
3. Align on best practices for reporting methods and results for aSyn PFF protein characterization in publications and presentations.

To keep the discussions focused on the above challenges, we shall refrain from discussing (1) the relevance of this model to study PD pathology and progression, (2) which type of aSyn aggregate is the pathological form, and (3) downstream use of the aSyn PFF protein to generate the in vivo or in vitro model. ASAP and MJFF realize the importance of these topics and will schedule additional workshops on these topics.

WORKSHOP DISCUSSION SUMMARY

1. PART 1: RECOMMENDATIONS FOR QUALITY CONTROL OF THE ASYN PFF PROTEIN

Monomer Generation

- Source of expression plasmid
  - Most groups use mouse aSyn PFFs because it seeds better than human aSyn PFFs in WT mice and rats.
  - Most groups use WT aSyn sequence. Plasmids are generally kept in house and not widely shared. *MJFF strongly recommends researchers deposit their plasmids at Addgene and provide the accession number in their publications.*
- Measuring and removing endotoxin
  - Group consensus on the importance of this step, Methods differ from endotoxin removal kit (common) to chromatography.
- Purify monomeric protein

    - Most groups use boiling and a form of chromatography (cation exchange, anion exchange, reverse phase, size exclusion)
        - Reverse phase chromatography does remove endotoxins but the end result is a lyophilized protein and when you resolubilize the protein you need to be very careful to monitor/control pH.
        - Using ion exchange chromatography only may affect the protein's aggregation propensity if there are contaminants while purifying the protein. Always check the protein purity, absence of preformed aggregates, and then check chemical integrity. Be sure to check stability over time and do not overload the column.
- QC monomeric protein
    - Most groups include SDS-PAGE to ensure purity of aSyn without cleavage products (<14 kDa).
    - It is important to keep monomer on ice and spin at 20,000*g* before to sediment any aggregated protein before using the supernatant containing monomer in experiments.
    - Some, but not all, also incorporate sedimentation and Thioflavin T assay alongside aggregated sample. Less common methods include TEM, dynamic light scattering, pyrotoxins, nucleic acid detection, mass spec, analytical ultracentrifugation, analytical RP-HPLC
    - It was recommended to check that the monomeric protein is not cleaved/truncated, and whether there is methionine oxidation. Mass spectrometry was recommended for this analysis.
    - For those using a commercial source of protein, there may be lot-to-lot variability and groups should always QC in house rather than relying on the manufacturer reports. This QC should include mass spectrometry, SDS-PAGE, and sedimentation assay to check for spontaneous aggregates.
        - Storing monomeric protein at a high concentration (>10 mg/mL) can also help with later PFF formation at 5 mg/mL. Often, after freeze thaws, some aSyn aggregates and after spinning, the concentration of monomer is lower than before it was aliquoted and frozen. This can be an issue with commercial protein given their provided concentrations.
        - Commercial proteins may also include an unspecified tag. This should be checked as it may affect aggregation/pathogenicity.
- Storage
    - Generally at -80C but duration varying from 6 months to indefinite. Aliquoting is recommended to avoid repeated freeze/thaws.

PFF Generation

- Generation
    - The buffer has a large impact on the formation of aggregates and the consistency between batches. Multiple groups report success using 150 mM KCl, 50 mM Tris-HCl at pH 7.4-7.5. It was recommended to NOT use PBS.
    - During sonication you may be releasing monomeric protein from the aggregates. Checking monomer-to-aggregate ratio is important. Hilal Lashuel reported a filter method for this use (citation here).
- Storage

    - Mixed reports of individuals storing aggregated material pre-sonication and post-sonication, but material is generally stored for ~1 year in 25 µL aliquots.
    - Once sonicated, there was variability in the amount of time individuals were willing to store the material prior to use. Some use immediately, RT for 12 hours, RT for 4 days, RT for 1 week, -80C (flash freeze in liquid N2). Most sonicate on day of use.
- Sonication
    - Different sonicators are used: bath (n=4), cup horn (n=3), probe (n=3).
    - Some report instability of the protein and amorphous aggregates with probe sonication, potentially due to temperature increase during sonication. Instability appears to affect pathogenicity, with a decrease in pathology over time. Others do not have this issue, indicating that methods for controlling temperature could be important.
    - Closed systems were recommended instead of probe tip for safety reasons.
- Concentration Measurement.
    - Mix of methods reported for measuring protein concentration: monomeric starting material using BSA, insoluble fraction post-aggregation using BSA, denatured aggregate sample using A280.
        - It was recommended to denature the aggregated protein using Guanidine HCl and measure protein concentration on that sample for the most accurate readout.
        - It was mentioned that reports on other proteins indicate that Guanidine HCl may not be breaking the aggregates into monomer, but rather smaller aggregates and increasing solubility. This has not been checked for aSyn but someone may want to do this.

Quality Control

- Groups are performing sedimentation assay, ThioflavinT assay, and circular dichroism; size analysis with electron microscopy, atomic force microscopy, and dynamic light scattering; and seeding propensity with in vitro and in vivo treatments.
    - Limited proteolysis profiling using Proteinase K is another method that can be used. This consists of subjecting the sonicated PFF sample to proteolysis by Proteinase K and analysis the kinetics of degradation using SDS gels. A specific banding pattern should be observed. Methods for this experiment are reported in publications by Ron Melki.
        - This would be a similar assay to the Thioflavin T assay to understand the different structures of aggregates. The benefit over Thioflavin T is that Proteinase K is not concentration dependent.
        - Acceptable banding patterns observed may differ between labs, making a standard benchmark difficult. Different sources of Proteinase K have been tested and do not cause variation in results. Hopefully results would be consistent between groups but this is not known.
    - As an additional step, analytical ultracentrifugation can also be used to understand aggregate properties like size, weight, concentration, etc. An alternative (cheaper) method for aggregate size is a sucrose gradient but it is unclear how sucrose may affect the protein.
- Recommendations on QC should be standardized but there is not one assay that gives you a full picture so you need multiple readouts.

- Begin with characterization of the monomeric protein. This needs to go beyond just the SDS-PAGE gel to enable understanding of cleavages, endotoxin, other contaminants, and spontaneous aggregates.
- Continue with the optimal method for measuring protein concentration.
- Incorporate multiple readouts to understand the structure of the aggregated material pre-sonication. The Thioflavin T and sedimentation assays are good, simple readouts. Limited proteolysis profiling using Proteinase K is also a good readout.
- Post-sonication, a quantitative or semi-quantitative readout of the size distribution (eg DLS) of the aggregates post-sonication is absolutely critical. Understanding the ratio of monomer-to-aggregate is also important and should be investigated.
- Pathogenicity can only be confirmed with pilot studies. In vitro studies, however, do not always predict the pathogenicity observed in vivo. For in vitro studies, primary neuron cultures are successful whereas immortalized cell lines are more difficult to seed. NeuN and pS129 aSyn are the common readouts of pathology.

2. PART 2: RECOMMENDATIONS FOR REPORTING METHODS AND RESULTS OF ASYN PFF CHARACTERIZATION

CATEGORY	MINIMUM TO REPORT	SUGGESTED TO REPORT
Details and Quality Control Assays of Starting Materials (monomer and fibrils pre-sonication)		
Source	vendor vs generated in-house vs collaborator	if generated in-house, provide methods including α-syn plasmid sequence
α-syn species	human vs mouse vs rat	
Quality control assay for structure/size	structure/size confirmed by TEM or DLS	TEM or DLS method details
Quality control assay for pelletability		Pelletability confirmed by sedimentation assay*
Quality control assay for amyloid secondary structure		amyloid structure confirmed by Thioflavin T*
Quality control assay for endotoxin levels	confirm <0.05 endotoxin units per 1 μg of protein	
Details and Quality Control Assays for Day-of Use Materials (*monomer and fibrils post-sonication)*		
Method used for sonication	type of sonicator (probe vs cup horn)	sonication parameters
Fibril size distribution	fibril size distribution (range, mean, proportion ≤ 50nm) and how determined (TEM or DLS)	images and/or graph of distribution
Working concentration of fibrils/monomer used	volume and concentration added to culture or injected in vivo	
Seeding efficiency confirmed in pilot study		pSyn accumulation confirmed under experimental conditions

Additional comments

- Individuals should be providing the aSyn sequence in their publications.
- QC on the monomer sample needs to be reported.
- Regarding the reporting of aggregate size post-sonication - quantitation should be required as providing one EM panel may be misleading.
- Better reporting of the concentration (method and result) of the aggregated protein is needed.

STANDARDIZING THE SYNUCLEIN PFF MODEL FOR PARKINSONS DISEASE

PART 2: UNDERSTANDING THE MODEL

MJFF/ASAP WORKSHOP – JULY 19, 2022

WORKSHOP GOALS

The alpha-synuclein (aSyn) PFF model is a widely used model of Parkinson's disease (PD). However, as with all models, there are aspects of PD pathology that may or may not be replicated. Therefore, a clear understanding of the appropriate readouts to use in the model to link the pathology to what is seen in patients is key.

The goal of this workshop is to

1. Review and recommend various readouts to understand the complexity of synuclein pathology.
2. Discuss outcome measures for understanding the functional implication of aSyn pathology.

WORKSHOP DISCUSSION SUMMARY

3. PART 1: METHODS USED TO CHARACTERIZE ASYN PATHOLOGY IN THE PFF MODEL

Investigating synuclein pathology

- The vast majority of folks report using pS129 aSyn as their primary readout of pathology. Second most common readout was insoluble/PK-resistant synuclein. Less frequently used readouts of synuclein pathology include synuclein aggregates or pSyn* (first reported here).
    - Regarding pS129 aSyn antibodies – challenges have been reported in the field. A paper on BioRxiv by Hilal Lashuel notes issues using the Abcam EP1536Y antibody (the most commonly used antibody) for immunocytochemistry. Abcam also removed some immunostaining applications from the approved applications list on the antibody product page. However, Laura Volpicelli-Daley noted that she has not experienced these issues as it appears to work for IHC/ICC and the signal is absent in synuclein KO samples. Jeff Kordower did note that he had some issues but these were resolved when switching from a PBS buffer to a Tris buffer.
    - Regarding total aSyn antibodies for use in detecting insoluble or PK-resistant aSyn – most groups report using the Syn1 antibody from BD. Laura Volpicelli-Daley says she's moved away from this antibody (now uses a polyclonal rabbit antibody from Abcam ab51252) because she was signing signal in synuclein KO tissue. Others may want to double check that their signal is specific when using the Syn1 antibody by using alpha-synuclein knockout lysates/tissue.

- It was mentioned that pS129 aSyn in cell culture systems isn't always indicative of pathology as this readout is present without PFF treatment. If you're seeing an increase in pS129 aSyn in your in vitro system, you should validate this with triton extraction to make sure it is insoluble and pathological.
- There was lengthy discussion around reports that some of the pS129 aSyn antibodies are showing a background nuclear stain which can increase in relation to synuclein pathology burned. This nuclear background stain was corroborated by 5+ groups on the call. While researchers/students can learn to distinguish the nuclear background stain from the specific cytoplasmic stain when using methods like stereology, this background can present a confound for other methods like analyzing percent area occupied using Fiji for looking at synuclein pathology. It was suggested that this might be antibody-specific and could be avoided by using other antibodies, but the challenge seems to persist across at least a few antibodies/labs.
- ***Key Takeaway – Antibody selection and validation is crucial as background staining is not infrequent with synuclein and phospho-synuclein antibodies. This background can contribute to misleading results or present as a confound when quantifying pathology.***

Challenges in investigating synuclein pathology

- Two main themes emerged regarding challenges in looking at different forms of synuclein pathology. These include measuring synuclein pathology pre-inclusion formation and analyzing different forms of synuclein pathology (including truncations, other post-translational modifications, etc). Both of these challenges can be attributed to a lack of available antibodies in addition to difficulty in identifying positive-control tissue.
    - Irene Griswold-Prenner noted that she has a spectrum of antibodies against nitrated synuclein species and they are willing to share samples with groups to help test specificity. Contact Irene (igriswold@nitrasetx.com) for more information.

4. PART 2: METHODS USED TO UNDERSTAND THE FUNCTIONAL CONSEQUENCE OF ASYN PATHOLOGY IN THE PFF MODEL.

In Vitro Model

- Most groups are using cell death as the primary readout of the functional consequence of synuclein pathology, with some other investigation into mitochondrial dysfunction, synaptic changes, etc. Cell death is reported across different cell model systems, including primary neurons from mice/humans, iPSCs, iPSC-derived dopamine neurons, etc.
- There was lengthy discussion around using cell death as the primary/only readout with regards to the heavy reliance on this readout and relevance of this readout to the disease. While some say this should be a central readout due to the fact that cell death occurs in patients and you can reliably observe cell death in the in vitro PFF model once the correct culture conditions are established, others are saying that it should not be the sole readout and the system should be designed to better mirror the patient condition that has progressive and selective cell death (in contrast to tailoring your in vitro system to immediate, aggressive cell loss).
    - For groups that want strong cell death phenotypes, Ted Dawson says this is heavily dependent on the methods you use to culture cells and can be reliably observed if glial overgrowth is inhibited, neuronal nitric oxide synthase is present, etc. Some differences in

this phenotype reported across groups is most likely attributed to differences in cell culture conditions and abundance of astrocytes in the culture.
    - Using an astrocyte inhibitor may increase reliability of cell death phenotypes. This can be done using 5-FDU:
        - 24hrs after seeding, replace half the media with fresh media + 20uM 5-FDU (final working concentration 10uM). Change half of the media ever 3-4 days without adding additional 5-FDU (see Dawson publications for protocol).
  - The general consensus was that, as with all models, you may need to tailor your experimental design to emphasize the pathology you want to observe/model.
- Synaptic changes (presynaptic loss, spine loss, etc) were also discussed at length. There was general consensus that this is a very interesting readout in cell cultures models since it is also observed as an early readout in animal models and is present in patients. More work needs to be done, however, to further understand this readout (for example, to understand the time course of presynaptic vs postsynaptic changes and using co-culture systems to understand the role of glia in this process). Also, this readout is time consuming for quantitation and not high-throughput.
- Throughout the discussion, it was noted that the method used for culturing cells can significantly impact the pathology observed. Even if the type of cell is the same between labs, differences in culture conditions can greatly impact the pathological readouts. In addition, the choice of brain region for primary neuron cultures (hippocampal vs cortical vs other) can impact your model as different neuron types expression synuclein at different levels.
  - Laura Volpicelli-Daley referenced a methods paper she published with Kelvin Luk (link here) that provides a protocol for using PFFs in primary cell cultures to try and help standardize this in the field. Please note, this protocol does not include inhibitors of astrocytes (see above mention of 5-FDU).
- Emphasis was also placed on understanding the time course of pathology and relation of the functional readouts to synuclein pathology load. Functional readouts should be evaluated as a time course against days in culture, PFF treatment, etc and correlated with different readouts of synuclein pathology. A recent report by Hilal Lashuel suggested that neuronal pathology following PFF treatment doesn't occur until you see interactions between synuclein and membranous organelles.
- ***Key Takeaway 1 – When using in vitro PFF models, it is important to understand and report the cell culture conditions to enable clear interpretation of the results and allow others in the field to adopt the model system.***
- ***Key Takeaway 2 – It is important to understand the time course of your readouts and relate this back to synuclein pathology to form a deeper understanding of the mechanism and biological underpinnings of your observations.***

In Vivo Model

- Many groups are focusing on readouts of nigrostriatal degeneration, including striatal neurochemistry/synaptic changes and neuronal loss in the substantia nigra. Many groups are then using behavioral tests for motor dysfunction to further understand the implication of this degeneration. Inflammation readouts are also quite common, with most focus on Iba1-related readouts.

- Most discussion focused around issues in robustness around readouts for motor deficits in this model. There was clear consensus that the nigrostriatal degeneration in the aSyn PFF model is less aggressive than neurotoxin models, with groups reporting an average of 40-60% nigral cell loss. This more moderate lesion leads to difficulties in detecting motor impairments, issues in reliably detecting motor deficits across cohorts, and a need for large group sizes (consensus that 15-20 animals/group are needed). More sensitive test of motor impairments are needed to detect deficits in the PFF model.
    - Marina Romero-Ramos says she sees hyperreactivity at early timepoints that appear to be attributed to compensatory changes in striatal dopamine release in the model, but it is generally difficult to see clear motor phenotypes using traditional tests.
    - Sheila Fleming says that she sees consistent deficits that are Ldopa-reversible in rats injected with PFFs in the striatum when using the movement initiation test but you need 60% dopamine cell loss for this readout. There is also good consistency across cohorts in decreased investigation in object recognition tests in this model.
    - Jim Surmeier also emphasized the point that the exact injection location in the striatum is important for reliable behavioral readouts. The restricted volume of toxicity in the PFF model and the difference in coordinates across labs could be contributing to some of the variability. Although most groups report "dorsolateral injection", the pre/post-commisural location will impact the amount of degeneration and resulting behavioral phenotypes.
        - Caryl Sortwell's group performed multiple injections in the rat striatum to cover a larger area (AP +1.6, ML +2.0, DV -4.0; AP +0.1, ML +4.2, DV -5.0).
    - In general, groups felt that behavioral tests were important to assess, even if they can be difficult to measure and require larger group sizes.
- There was also discussion around the observation that synuclein pathology in different brain regions can be transient, with loss of pS129 aSyn signal at later timepoints. This was reported by Nathalie Van Den Berge in the following gut injection, Patrik Brundin following olfactory bulb injection, Ted Dawson following gut injection, and Jim Surmeier following brainstem injection. This is also reported in other model systems as Deniz Kirik published a loss of aSyn pathology 12 months post-injection with AAV aSyn. This could be due to the cell's capacity to clear pathology over time or subtle cell death that does not reach significance when quantified.
- There was also a methods discussion around the best ways to quantify nigral neurons for cell loss.
    - TH can be downregulated and is not a good readout for frank cell death. Marina Romero-Ramos suggested that VMAT and DAT are markers for dopamine neurons that are less susceptible to downregulation than TH. However, they can still be downregulated to an extent so they might not be the best readout for frank cell loss.
    - NeuN is often used but a report from the Greenamyre lab indicates that it might not be a reliable readout for dopamine neurons.
    - Cresyl/Nissl are used but are difficult as they are not neuron-specific stains and require a trained eye to differentiate neuronal vs non-neuronal cells.
    - Fluorogold was also floated as an option but this will only stain a subset of cells and may not be compatible with all fixation methods.
    - Virginia Lee suggested using antibodies to phosphorylation-independent neurofilament to stain cells.
    - Caryl Sortwell recommended HuC as a good cytoplasmic neuronal marker.

- ***Key Takeaway – When evaluating pathology (especially nigrostriatal degeneration and motor dysfunction) in the aSyn PFF in vivo model, injection location is critical. Exact injection coordinates should be reported in publications, along with information on PFF dose and rodent strain/age used, to facilitate replication and enable interpretation of the results as differences in readouts across labs could be due to differences in synuclein pathology levels or location.***

STANDARDIZING THE SYNUCLEIN PFF MODEL FOR PARKINSONS DISEASE

PART 3: CHOOSING THE INJECTATE

MJFF/ASAP WORKSHOP – AUGUST 3, 2022

WORKSHOP GOALS

While many have chosen to use recombinantly generated alpha-synuclein PFFs to model Parkinson's disease (PD) in vitro and in vivo, others have decided to use sources of synuclein aggregates that are derived from patient-derived material. This includes patient brain-derived material as well as synuclein fibrils generated through RT-QuIC/PMCA seeding reactions using patient biosamples.

The goal of this workshop is to discuss important considerations when using various protein sources to generate a synuclein model, specifically recombinant aSyn PFFs, patient brain-derived material, and fibrils generated through RT-QuIC/PMCA seeding experiments (SAA-fibrils).

KEY TAKEAWAYS

- When using samples from patients for Lewy body extracts or SAA-fibrils, you should carefully select and characterize the samples to reduce variability. Select patients with the same disease, disease severity, clinical phenotype, and pathology. Use samples from the same brain region and characterize them well to understand and quantify the amount of synuclein and other co-pathology proteins (tau, aBeta). Doing this should give you very little variability between materials from different patients within a disease.
- When using SAA-fibrils, troubleshooting may be needed in the SAA setup to determine the right ratio of patient sample to monomeric protein. It is also recommended to stick with one passage and use patient samples to seed new lots (rather than using SAA-fibrils from a previous run). This will increase consistency and prevent a shift in aggregate properties. Ideally, future experiments could develop methods to passage SAA fibrils continually without losing the characteristics of the original SAA fibrils, but more work is needed to establish a reliable method that can be used across labs .
- The methods used to isolate Lewy body extracts or process tissue for SAAs will bias you towards a certain population of aggregates. For instance, selecting brain tissue may bias you towards whatever insoluble aggregates are still present in the end-stage disease brain once substantial cell death and degeneration have occurred (whereas CSF may bias more towards the soluble, released pool of aggregates). Detergents and tissue processing methods will similarly drive you towards specific populations of aggregates.
- Although there may be some variability introduced by patient selection when using Lewy body extracts or SAA fibrils, there is also quite a bit of variability when using recombinant aSyn PFFs and

the samples appear to harbor a more heterogenous mix of aggregates than SAA-fibrils. Therefore, concerns about variability shouldn't lead one to reject Lewy body extracts or SAA-fibrils outright.

- We still don't know what the "toxic" species of synuclein aggregates is within the disease. The case can be made for/against each one of the different injectate options in this regard:
    - Recombinant PFFs – Clear differences exist between the properties of recombinant PFFs vs Lewy body extracts or SAA-fibrils, and recombinant PFF preps contain a heterogenous mix of aggregates. However, they are easy to characterize, the PFFs are consistent and generate consistent phenotypes when protocols are followed carefully, the PFFs are easy to ship, and sourcing recombinant protein is easier than patient samples.
    - Lewy body extracts – Extracts contain an abundance of proteins other than aSyn aggregates that might influence the pathogenicity of samples and the synuclein aggregates selected for in these samples are insoluble and from the end stage of disease when significant cell loss has occurred. However, the extracts are pretty consistent across patients and likely contain a mix of different forms of aSyn aggregates that are present within the patient brain.
    - SAA-fibrils from brain extracts or CSF – SAA-fibrils generally bias towards a single aggregate type or a less diverse set of aggregates that are more prone to template in the reaction; the disease relevance of this is unclear. Using brain extract as the starting material biases you towards an insoluble aggregate present within the remaining cells at the end stage of disease whereas the use of CSF presents challenges in the low concentration of seeds present and biases you towards a secreted aggregate. The benefit of this injectate is that it is derived from patients but doesn't present the purity/concentration issues of Lewy body extracts.
- It is very clear that future research is critical to identify the toxic species of synuclein aggregate(s) to determine the source of injectate and characteristics of the synuclein aggregates that best recapitulate PD phenotypes in models.

WORKSHOP PRESENTATION AND DISCUSSION SUMMARY

Comparing recombinant aSyn PFFs vs patient Lewy Body extracts vs SAA-fibrils

- Many groups reported differences in the biochemical/biophysical properties of recombinant PFFs vs SAA-fibrils. Most use PK digestion coupled with SDS-PAGE to visualize differences in banding pattern between the different materials. TEM has indicated differences between PFFs and SAA-fibrils at a high level (eg twisted structure) and cryoEM is a good method for visualizing the different forms of beta-sheet structures in aSyn aggregates seeded from patient material (ie SAA-fibrils).
    - Regarding the structure of aSyn aggregates, it seems the 6ssx structure for aSyn aggregates is coming up relatively frequently in CryoEM studies.
- The Virginia Lee lab presented on their work comparing different sources of injectate (publication here). Their study compared recombinant human aSyn PFFs to Lewy Body extracts from PDD or AD patients and SAA-generated fibrils from brain homogenate from PDD or AD patients.
    - They noted that the human-derived material displayed different pS129 aSyn staining patterns compared to recombinant PFFs in primary mouse neuronal cultures:

        - Recombinant PFFs = ~95% of pS129 aSyn staining is present in the neurites, similar to what is seen in PD brains.
        - Lewy body extracts and SAA-fibrils = pS129 aSyn staining is split 50/50 between neurites and soma. This distribution of pS129 aSyn staining was consistent across timepoints (neurite pathology did not precede somatic pathology).
    - A similar pattern was observed when injecting the materials in the WT mouse striatum:
        - Lewy body extracts resulted in more somatic pathology at 6+ months but pathology was generally low at 3 months, indicating a different timecourse of pathology when using patient-derived material vs recombinant PFFs.
        - In mice, the proportion of somatic-to-neuritic pS129 aSyn pathology with Lewy body extracts was not as dramatic as in the cell culture system. Neuritic pathology is more evident when injected in mice compared to when added to primary neurons.
    - It was noted that the increased proportion of somatic pathology when Lewy body extracts or SAA-fibrils could be due to the tissue processing methods biasing synuclein aggregate recovery more towards the stable population present in somatic Lewy bodies rather than another population that is more soluble or neurite-localized.
- George Tofaris noted that their lab doesn't see big differences in somatic vs neuritic pathology when comparing their PD/MSA SAA-fibrils to recombinant PFFs in human dopamine neurons (publication here). However, the patient-derived material does induce more toxicity than PFFs.
- As patient Lewy body extracts and SAA-fibrils will contain aggregates of human aSyn, it is important to consider species homology differences when applying to mouse primary cultures or injecting into WT rodents as pathology might be less robust when using human aSyn aggregates to seed pathology in endogenous mouse synuclein.
    - It may be worth considering using human cell culture systems and rodents expressing human synuclein for these studies.
    - Caution should be taken when attempting to generate SAA-fibrils using recombinant mouse monomeric aSyn substrate protein as sequence differences between the human and mouse protein may lead to aggregates that don't faithfully recapitulate the starting material.

Variability when using Lewy body extracts or SAA-fibrils

- In the Virginia Lee lab presentation/publication, Lewy body extracts and SAA-fibrils were obtained from patients with PDD and AD (3 patients per disease). Materials from both diseases presented similarly in terms of pathogenicity when the samples are well characterized (measure levels of aSyn, tau, etc) and you control for the level of aSyn in the sample (quantified with ELISA and WB).
    - Tim Bartels echoed this notion that samples must be well characterized and added that you might want to quantify insoluble aSyn via ELISA.
- Others report differences between diseases. Tim Bartels overviewed his work using aggregates from PD, MSA, and DLB patients to induce pathology in a cell culture system using human iPSCs that overexpress aSyn. He notes differences between diseases in pS129 aSyn inclusions and the timecourse of pS129 aSyn pathology.
    - Similarly, George Tofaris sees differences in toxicity between aSyn aggregates isolated from MSA vs PD. In studies looking at the interaction between PD vs MSA patient-derived

aggregates and different proteins (publication here), he is seeing many shared interactors between the diseases but key differences in a few of the proteins that could be mediating differences in the properties/toxicity of the PD vs MSA-derived aggregates.

- The source of human aggregates may also account for variability. For example, if Lewy bodies are purified from PD cortex this may result in consistent phenotypes. However, when SAA is performed using CSF from PD or DLB patients, there may be more variability (potentially because the CSF aggregates are a more heterogeneous population).
    - Many report little variation in pathogenicity (pS129 aSyn) of SAA fibrils derived from brain samples between patients within a disease if the patients have similar pathology and disease duration (voiced by Tim Bartels, Virginia Lee, Ted Dawson). If the patients selected are very heterogenous with varied disease duration and pathology, then there will be high variation between the patients
    - Andy West used SAA to generate fibrils from postmortem CSF and saw heterogeneous phenotypes with respect to abundance of pS129 aSyn aggregates and microglial activation. He also noted the characteristics of the SAA fibrils from postmortem CSF differed from SAA fibrils generated from brain homogenate from the same patient.
- Much more research is needed to understand the toxic species of synuclein aggregates and compare the phenotypes from SAA fibrils generated from different samples- brain extracts, extracts from different regions of the brain, CSF, etc.
- It is important to note that there may be more variation between patients when looking at readouts other than pS129 aSyn, especially readouts that are more detailed or looking at other pathways/pathology.
- Tim Bartels presented on his work that demonstrates an impact of brain region on the pathogenicity of SAA-fibrils. When characterizing the brain homogenates, different banding patterns are observed in PK-digestion SD-PAGE, the SAA profiles are different, and the SAA-fibrils induce pathology differently. Although the reason for this is unclear, the hypothesis is that it is due to the interplay of the immune system.