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Investigating the Potential Role of α-Synuclein in Tau Aggregation and Toxicity

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Investigating the Potential Role of α-Synuclein in Tau Aggregation and Toxicity

by

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Investigating the Potential Role of α-Synuclein in Tau Aggregation and Toxicity

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A group of neurodegenerative diseases that are pathologically characterized by the presence of intracellular abnormal aggregation of α-Synuclein (α-Syn) in Lewy bodies (LBs) and Lewy neurites (LNs), are collectively known as synucleinopathies. Even so, tau protein pathology is abundantly found in these diseases. Apart from Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), LBs and LNs have been reported in Alzheimer's disease (AD) patients as well. Both α-Syn and tau can exist as polymorphic aggregates, and this phenomenon has been widely studied, mostly in their fibrillar assemblies. Growing evidence suggests that intermediate metastable oligomeric assemblies of several amyloidogenic proteins, including α-Syn and tau are actual neurotoxic species. However, little is known about the structural and functional heterogeneity among α-Syn oligomers occurring in different diseases. Moreover, the functional crosstalk between these toxic oligomers has not been scrupulously studied. Here, by using biochemical, biophysical and cell-based techniques, I have studied the structural and functional diversity of distinct recombinant α-Syn oligomers, prepared by modifying the protein with two physiological inducers, dopamine (DA) and docosahexaenoic acid (DHA). The two recombinant α-Syn oligomers differed in aggregate size, conformation, sensitivity to proteinase K digestion, tryptic digestion and toxicity, suggesting them as distinct α-Syn oligomeric strains. I have also analyzed brain-derived α-Syn oligomers (BDSOs) from AD, DLB and PD brain tissues. I observed that disease associated BDSOs were diverse in their functional properties. Notably, they can be uptaken via gap junction protein Cx50 in the primary neurons, thus suggesting a unique mechanism that might be involved in the oligomers mediated toxicity. Additionally, both recombinant and brain-derived α-Syn oligomeric strains effectively cross-seeded tau aggregates with diverse biochemical, biophysical and biological properties. Interestingly, BDSOs cross-seeded tau aggregates were more potent seeds causing cellular tau aggregation than the ones cross-seeded with recombinant α-Syn oligomeric strains. The findings here represent a significant step to elucidate the toxic interplay between α-Syn oligomeric strains and tau, altering the aggregation profiles and nature of the amyloid deposits. This will lay the groundwork for more successful therapeutic interventions by targeting multiple candidate molecules, such as α-Syn and tau in diseases.

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LIST OF ABBREVIATIONS

PD: Parkinson's Disease

PDD: Parkinson's Disease with Dementia

DLB: Dementia with Lewy Bodies

MSA: Multiple System Atrophy

AD: Alzheimer's Disease

MCI: Mild Cognitive Impairment

TSE: Transmissible Spongiform Encephalopathy

PSP: Progressive Supranuclear Palsy

FTDP-17: FrontoTemporal lobar Dementia with Parkinsonism linked to chromosome-17 (FTDP-17)

CBD: CorticoBasal Degeneration

PiD: Pick's Disease

TBI: Traumatic Brain Injury

GC : Gaucher's Disease

α-Syn: α-Synuclein

BDSO: Brain Derived α-Synuclein Oligomers

MTBR: MicroTubule Binding Region

NFT: Neurofibrillary Tangles

LB: Lewy body

LN: Lewy Neurite

TSE: Transmissible Spongiform Encephalopathy

PTM: Post-Translational Modifications

SUMO: Small Ubiquitin-like MOdifier protein

UCH-L1: Ubiquitin Carboxy-terminal Hydrolase L1

CHIP: C-terminus of Hsp70 interacting protein

SIAH: Seven In Absentia

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

NFT: NeuroFibrillary Tangles

NAC: Non-Amyloid Component of plaques

MAPT: MicroTubule Associated Protein Tau

PQC: Protein Quality Control

DA: Dopamine

aCSF: Artificial Cerebrospinal Fluid

PUFA: Poly-Unsaturated Fatty Acid

DHA: DocosaHexaenoic Acid

GAG: GlycosAminoGlycan

HSPG: Heparan sulfate proteoglycans

RAGE: Receptor for Advance Glycation End products

SNCA: Synuclein Alpha

MAP2: Microtubule Associated Protein 2

Cx50: Connexin50

LAG3: Lymphocyte-Activation Gene 3

PINK1: PTEN-induced kinase-1

LRRK2: Leucine-rich repeat kinase 2

IP: Immunoprecipitation

PK: Proteinase K

CD: Circular Dichroism

FTIR: Fourier transform infra-red microscopy

LC-MS: Liquid chromatography-mass spectroscopy

PRM: Parallel Reaction Monitoring

Bis-ANS: 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt

ThT: Thioflavin T

AFM: Atomic Force Microscopy

LDH: Lactate Dehydrogenase

PET: Positron Emission Tomography

fMRI: functional Magnetic Resonance Imaging

EGFP: Enhanced Green Fluorescent Protein

SEC: Size Exclusion Chromatography (SEC)

EM: Electron Microscopy (EM)

Cryo-EM: Cryo-Electron Microscopy

NMR: Nuclear Magnetic Resonance

CHAPTER 1: INTRODUCTION

BACKGROUND

Neurodegenerative diseases are a group of devastating diseases that affect day to day life by impairing memory and/or movement, thus significantly deteriorating the quality of life (Przedborski, Vila et al. 2003). With increasing life expectancy, the incidences of these diseases are increasing, imposing a tremendous burden on socio-economic status world-wide (Masuda-Suzukake, Nonaka et al. 2013). Apart from the clinical symptoms, these degenerative diseases are pathologically characterized by the presence of proteinaceous aggregates. It is well established that protein misfolding and its subsequent aggregation leading to abnormal accumulation is the key event of neurodegeneration (Ross and Poirier 2005). However, the mechanistic details of abnormal protein aggregation in neurodegenerative diseases remains still elusive.

SYNUCLEINOPATHIES

A group of neurodegenerative diseases, which are pathologically characterized by the presence of intracellular accumulation of the protein, α-Synuclein (α-Syn), are collectively known as synucleinopathies (Marti, Tolosa et al. 2003). The pathological hallmarks of synucleinopathies are the abnormal depostion of fibrillar α-Syn protein into Lewy bodies (LBs) and dystrophic Lewy neurites (LNs). These LBs and LNs are observed in the selected populations of neurons and glial cells in the affected brain regions (Jellinger 2003). The three main synucleinopathies include Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). In PD and DLB, intraneuronal LBs and LNs are observed, whereas in MSA, the pathological hallmark is oligodendroglial

inclusions of α-Syn (**Figure 1.1**). Although, PD is primarily a motor disorder accompanied by disturbed sleep pattern and non-autonomic dysfunctions, however, later in the disease stage, patients do also experience dementia. In contrast, DLB is mainly viewed as a progressive dementia with hallucinations and sleep disorder. MSA is a progressive but sporadic neurological disorder accompanied by cerebellar and autonomic dysfunctions

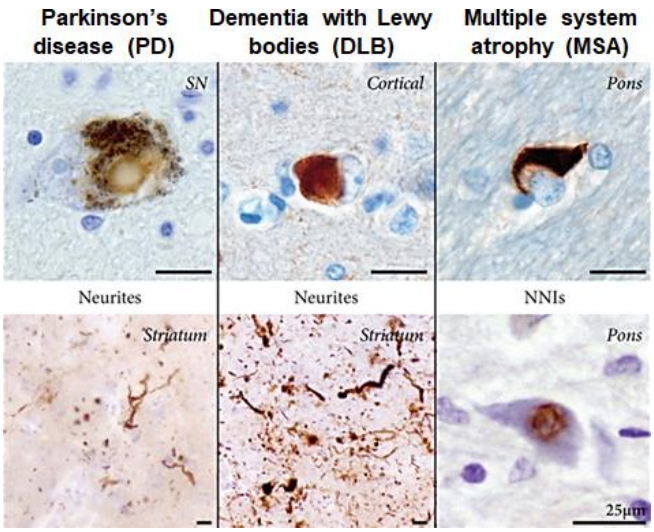

Figure 1. 1 Pathological α-Syn protein aggregates in synucleinopathies.
α-Syn inclusions in the three major synucleinopathies, Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA): intraneuronal Lewy bodies (LBs) and Lewy neurites (LNs) in PD and DLB and glial cytoplasmic inclusions (GCIs) and neuronal intranuclear inclusions (NNIs) in MSA (McCann, Stevens et al. 2014)

(Gai, Power et al. 1998, Spillantini, Crowther et al. 1998). Despite their differences in clinical manifestations, all the three diseases contain α-Syn protein aggregation in common (Ono 2017). However, as there is a huge overlap between multiple neurodegenerative diseases, co-morbidity has become a common event. Additionally, there are several other disorders such as Parkinson's disease associated with dementia (PDD), and a rare genetically inherited metabolic disease, Gaucher's disease (GD), where Lewy pathology has been reported, thus considering them as synucleinopathies. Moreover, axonal spheroids consisting of α-Syn are the hallmark aggregates of neurodegeneration with brain iron

accumulation disorders (NBIA), which are also considered as synuncleinopathies (Barker and Williams-Gray 2016). Among the above mentioned Synucleinopathies, DLB, PD and PDD show abundant tau protein pathology. On the other hand, Alzheimer's disease (AD) is the most common form of dementia that has been commonly viewed by the aggregation of hyperphosphorylated tau into neurofibrillary tangles (NFTs) and senile plaques of amyloid β protein. Interestingly a significant amount of LBs and LNs have been reported in AD patients (Koehler, Stransky et al. 2013, Mattsson, Insel et al. 2013), thus categorizing AD also as a synucleinopathy (Uchikado, Lin et al. 2006). Additionally, α-Syn protein was detected in the cerebrospinal fluid (CSF) of patients with AD and mild cognitive impairment (MCI) (Korff, Liu et al. 2013). In a transgenic mouse model expressing amyloid precursor protein, α-Syn protein was detected in the amyloid plaques, indicating a crosstalk between different amyloid proteins (Yang, Ueda et al. 2000).

ALPHA-SYN PROTEIN: STRUCTURE AND FUNCTION

α-Syn is a 14 kDa (140-amino acid long) intrinsically disordered protein, which is abundantly present in the brain. It is a presynaptic protein but is also found in the cell soma and nucleus (Emanuele and Chieregatti 2015). This protein is mainly comprised of three regions: a lysine-rich amphipathic N-terminus region (containing a repeat of KTKEGV) that determines its interaction with membranes; a disordered negatively charged C-terminus region, responsible for its interaction with nuclear envelop and other proteins; and a highly hydrophobic middle region (61-95 amino acids long) (**Figure 1.2**) (Emanuele and Chieregatti 2015). Until now, 7 missense mutations have been identified in the N-terminal region of the protein (A30P, E46K, H50Q, G51D, A53T, A18T and A53E) that are associated with the familial forms of PD, DLB and MSA, although accounting for only

~10% of the total disease incidence (Kruger, Kuhn et al. 1998, Appel-Cresswell, Vilarino-Guell et al. 2013, Hoffman-Zacharska, Koziorowski et al. 2013, Lesage, Anheim et al. 2013, Proukakis, Dudzik et al. 2013, Pasanen, Myllykangas et al. 2014, Zhang, Xia et al. 2018). This middle region is known as Non-Amyloid-β Component of plaques (NAC) since its discovery from amyloid plaques of AD patients (Emanuele and Chieregatti 2015). The NAC region of α-Syn contributes to its aggregation, as the partial deletion of this

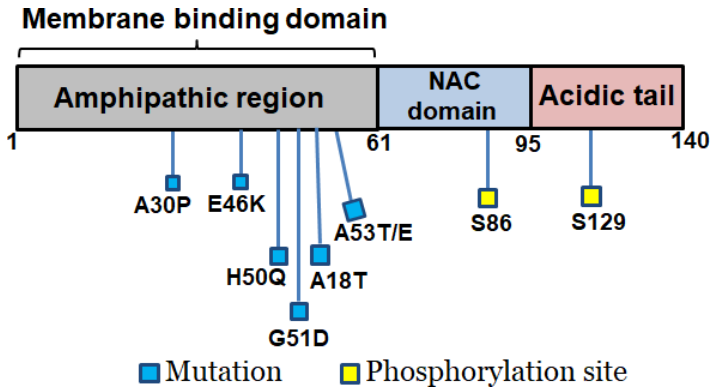

Figure 1. 2 Domain structure of α-Syn protein.
α-Syn contains an N-terminal amphipathic region, a middle domain of non-amyloid-β component (NAC) and an acidic tail at C-terminus. Until now, seven missense mutations have been identified. α-Syn protein Phosphorylation at serine 129 residue is associated with pathological α-Syn protein aggregation (Emmanuele and Chieregatti 2015)

region disrupted oligomerization and consequently fibrilization process of this protein. Due to its localization in the presynaptic vesicles, α-Syn is believed to regulate synaptic plasticity by regulating recycling of synaptic vesicles and neurotransmitter release (Burre 2015, Villar-Pique, Lopes da Fonseca et al. 2016, Goedert, Jakes et al. 2017). Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), is a large protein complex required for synaptic vesicle fusion and release of neurotransmitters in the synaptic cleft. It has been shown that α-Syn directly interacts with the SNARE protein, synaptobrevin-2, thereby, promoting SNARE complex assembly and clustering of synaptic vesicles (Burre, Sharma et al. 2010). Additionally, multiplications such as duplications and

triplications of SNCA gene encoding α-Syn protein are shown to be associated with familial forms of Synucleinopathies (Ingelsson 2016). Such multiplications can lead to the overexpression of the protein, resulting in its abnormal accumulation in the disease pathogenesis. In contrast, although mutant α-Syn protein aggregation is also observed in the familial forms of synucleinopathies, the pathophysiology caused by these aggregated mutant α-Syn protein is still unknown. While several mechanisms have been proposed for such neurotoxicity, one of the mechanisms is the variable propensity of mutant α-Syn to form aggregates (Flagmeier, Meisl et al. 2016). α-Syn is extensively modified by post-translational modifications including phosphorylation, ubiquitination, sumoylation, nitration, truncation, glycation and glycosylation. Such post-translational modifications contribute to the protein's function, degradation and even its aggregation propensity causing toxicity (Oueslati, Fournier et al. 2010).

Phosphorylation

The C-terminal region of α-Syn protein harbors most of the phosphorylation sites (Uversky and Eliezer 2009). LBs and LNs are profoundly comprised of α-Syn phosphorylated at serine 129 position (pS129). Mass spectrometry analysis of α-Syn aggregates isolated from the brain tissues of DLB patients showed extensive phosphorylation of α-Syn at serine 129 site (Fujiwara, Hasegawa et al. 2002). Thus, phospho-serine 129 has become a delineating feature of these pathological hallmarks. Phosphorylation at tyrosine 125 residue plays neuroprotective role against α-Syn aggregation, specifically oligomerization. Apart from this site, the other two phosphorylation sites observed are at tyrosine 133 and tyrosine 136 residues (Chen, Periquet et al. 2009). Phosphorylation states of α-Syn also modulate its interaction with

other proteins. In one study, a single domain binding antibody fragment, NbSyn87 was synthesized that bound to the C-terminal region of α-Syn near the 2 phosphorylation sites with nanomolar affinity. Phosphorylation of α-Syn at S129 site did not affect the binding efficiency of NbSyn87 to α-Syn significantly (Guilliams, El-Turk et al. 2013). Whereas, phosphorylation of α-Syn at tyrosine 125 decreases the binding affinity of NbSyn87 with α-Syn by approximately 400 times less (El Turk, De Genst et al. 2018). These observations indicate that although α-Syn phosphorylation at S129 is a pathological feature, phosphorylation at Y125 might play a role for its interaction with other proteins. Several kinases are shown to induce phosphorylation of α-Syn at serine 129 position, such as G-protein coupled receptor kinases (GRK1, 2, 5 and 6), casein kinase I and II, and polo-like kinases (PLK) (Chen, Zhao et al. 2019).

Ubiquitination and Sumoylation

Fibrillar aggregates of α-Syn is highly ubiquitinated in the LBs and LNs and such ubiquitination is a pathological event in synucleinopathies (Tofaris, Razzaq et al. 2003). It has been observed that the ubiquitination of α-Syn is unaffected by its mutations or phosphorylation status in PD pathogenesis (Nonaka, Iwatsubo et al. 2005). Ubiquitination of α-Syn can be caused by four E3- and E4-ubiquitin ligases, such as Parkin, seven in absentia (SIAH), C-terminus of Hsp70 interacting protein (CHIP) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) (Lee, Wheeler et al. 2008, Walden and Muqit 2017). In vitro protein sequence analysis demonstrated that α-Syn is ubiquitinated at Lys21, Lys23, Lys32 and Lys34 (Mund, Masuda-Suzukake et al. 2018). The primary modification of α-Syn by small ubiquitin-like modifier protein (SUMO) is primarily through monosumoylation process by SUMO1 protein (Dorval and Fraser 2006). A recent study

has shown that sumoylation of α-Syn directly promotes its aggregation with several fold higher effect on disease associated mutant α-Syn A30P, A53T and E46K compared to WT α-Syn protein. On the contrary, such sumoylation process was also shown to impair ubiquitination process of α-Syn, thus preventing its aggregation (Rott, Szargel et al. 2017).

Nitration

Damages caused by nitration and oxidation of α-Syn has been studied in PD pathogenesis. Nitration and oxidation of α-Syn result in its aggregation (Uversky 2007). α-Syn protein has four tyrosine residues: Y39, Y125, Y133 and Y136, which are the sites for nitration (Burai, Ait-Bouziad et al. 2015). It has been shown that specifically Y-39 nitration leads to enhanced oligomer formation of α-Syn. At the same time, mutation in this site causes increased fibril formation (Danielson, Held et al. 2009).

Truncation

Several naturally occurring truncated regions of α-Syn have been reported. Both N-terminal and C-terminal truncated variants have been found in human brain tissues (Muntane, Ferrer et al. 2012). Truncated variants of α-Syn were also detected from the brain tissues of PD patients accounting for almost 10-30 % of the total α-Syn in LBs (Li, West et al. 2005, Liu, Giasson et al. 2005). Caspase 1, a proinflammatory molecule has been shown to cleave full-length α-Syn protein at C-terminus, thus generating a 19-residue long variant (Ma, Yang et al. 2018). Several other enzymes such as calpain I, Neurosin, Cathepsin D, and Matrix metalloproteinase 3 are also shown to be involved in the truncation process of α-Syn protein (Zhang, Li et al. 2019). In vitro C-terminally truncated α-Syn showed enhanced aggregation with neurotoxicity (Periquet, Fulga et al. 2007). A

recent study has reported that both C- and N-terminal truncated variants of α-Syn induce fibril formation (Terada, Suzuki et al. 2018).

Glycation and Glycosylation

Glycation process of α-Syn is suggested to cross-link the protein, thus making it resistant to proteolysis. Such glycated α-Syn was observed in the substantia nigra and locus coeruleus of PD patients (Vicente Miranda and Outeiro 2010). Increased level of α-Syn glycation and the receptor for advance glycation end products (RAGE) have been found in PD patients as compared to healthy individuals (Guerrero, Vasudevaraju et al. 2013). On the other hand, glycosylation of α-Syn in the NAC region is shown to inhibit its aggregation process (Marotta, Cherwien et al. 2012).

TAU PROTEIN: STRUCTURE AND FUNCTION

Tau is a microtubule-binding protein encoded by MAPT gene consisting of 16 exons, amongst which 11 exons are expressed in the central nervous system (CNS) (Pittman, Fung et al. 2006). Alternative splicing of the exons present in MAPT gene produces 6 isoforms. The 3 isoforms containing exon contain four microtubule-binding domains (MTBD) that are known as tau 4R, whereas, the 3 isoforms lacking exon 10 contain three MTBD and are known as tau 3R. All the 6 isoforms are abundantly present in the CNS (Pittman, Fung et al. 2006). The longest tau isoform, 2N4R contains 2 N-terminal domains (N1 and N2), a proline-rich domain (PRD) containing 2 units, 4 MTBD regions and a short C-terminal region (Pedersen and Sigurdsson 2015). The structures of all tau isoforms along with their molecular weights are depicted in **Figure 1.3**. Tau protein is highly susceptible to phosphorylation at serine, threonine and tyrosine residues. Almost 30 or more phosphorylation sites have been discovered on each tau isoform associated with both

physiological and pathological functions(Wolfe 2012). In its native form, tau is a mediator of microtubule dynamics and is important for cellular health. The phosphorylation state of

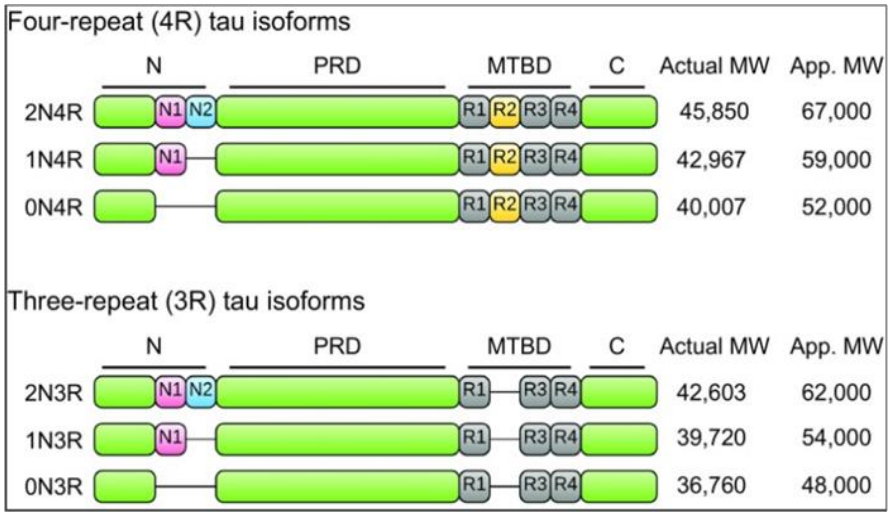

Figure 1. 3 Schematic representation of six isoforms of human tau protein showing domains structures.
The N-terminal region of tau is encoded by exons 2 and 3 that are either exclude (0N) or included forming 1N or 2N isoforms. The mid region is enriched in proline residues, called proline rich domain (PRD). Microtubule binding domain (MTBD) resulting from alternative splicing constitutes 3R and 4R tau. A common C-terminal end for all six isoforms. The actual molecular weight (MW) and apparent MW of each 3R and 4R tau isoform is given (Guo, Noble et al. 2017)

tau is mainly thought to control an essentially required dynamic equilibrium between tau and microtubules (Ballatore, Lee et al. 2007). The hyper-phosphorylated form of tau is known to form intraneuronal neurofibrillary tangles (NFTs) that destabilize microtubules and affect normal cellular processes (Zhang, Higuchi et al. 2004). However, other post-translational modifications on tau protein like, glycosylation, glycation, acetylation, ubiquitination, sumoylation and nitration have also been reported (Ballatore, Lee et al. 2007). Several neurodegenerative disorders are associated with the aggregates of tau. These disorders, collectively known as tauopathies include AD, frontotemporal lobar dementia with Parkinsonism linked to chromosome-17 (FTDP-17), progressive supranuclear palsy

(PSP), corticobasal degeneration (CBD), Pick's Disease (PiD), traumatic brain injury (TBI) (Ballatore, Lee et al. 2007, Gendron and Petrucelli 2009) and several others.

PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are also known as proteinopathies as they involve abnormal aggregation of amyloidogenic proteins. It is believed that over-production and impaired clearance mechanism of the cell are probably responsible for abnormal protein aggregation (Ross and Poirier 2004, Ross and Poirier 2005, Kumar, Sami et al. 2016). Under physiological condition, once a protein is synthesized into its primary amino acid sequence, it acquires a 3-dimensional conformation to achieve a fully functional state. A balance is constantly maintained between synthesis and degradation of the proteins to avoid any malfunction protein hampering cell viability. In this regard, Protein Quality Control (PQC) system plays significant role by rapidly removing undesirable proteins (improperly or partially folded, produced in excess or damaged proteins) and maintaining cellular homeostasis (Hartl, Bracher et al. 2011). Under unfavorable conditions, protein folding machineries and PQC fail to perform its proper surveillance function to remove undesirable proteins which leads to cell toxicity. These misfolded or partially folded proteins tend to aggregate by self-association and elongation that are rich in β-sheet contents arranged in specific patterns (Jahn and Radford 2008). In unfavorable conditions, monomeric protein might change its conformation, thus initiating its aggregation process. As the 2 monomeric units join, they form dimer which further continues to aggregate, forming oligomers which are mostly spherical in shape, proto-fibrils and ultimately giving rise to β-sheet rich large inclusion bodies (**Figure 1.4**).

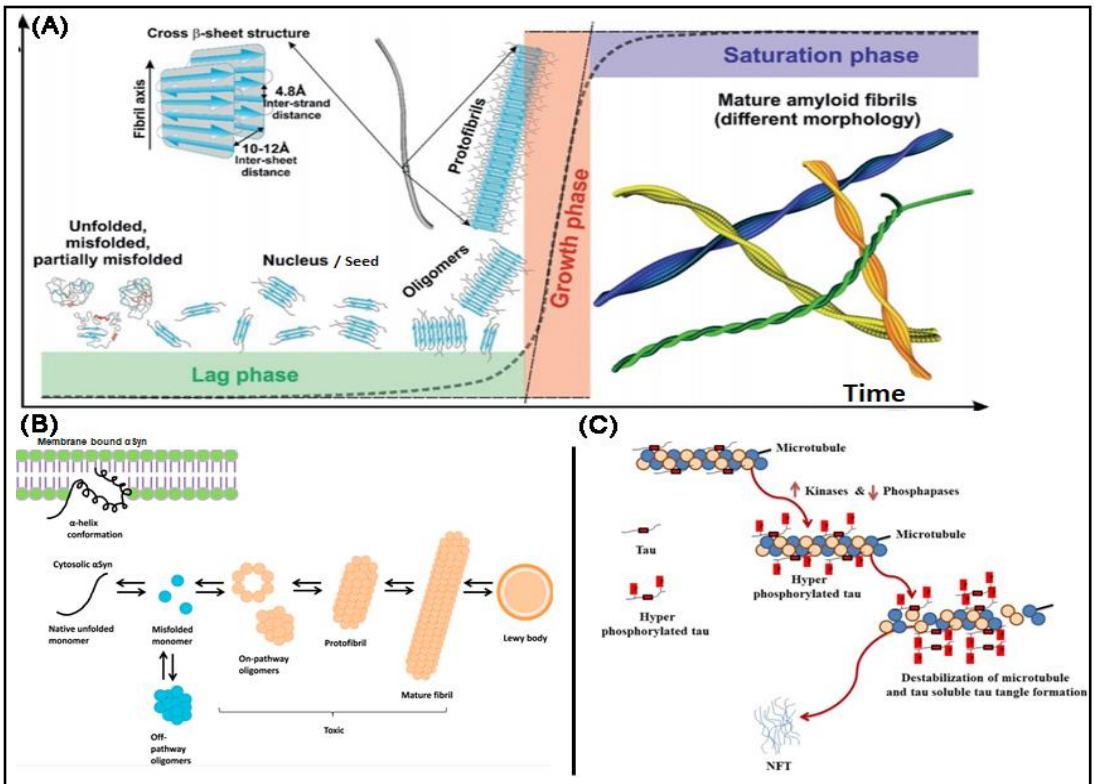

Figure 1. 4 Cascade of amyloid formation.
(**A**) Formation of ordered amyloid aggregation follows a typical sigmoidal curve with three phases. In the first lag phase, a nucleus is formed from unfolded, misfolded or partially folded peptides, which grows into oligomers by inter-molecular interaction. This is followed by a growth phase or log phase, where the aggregation process rapidly continues resulting in the formation of protofibrils and finally forming fibrils. The last phase is saturation phase, where mature fibrils of different morphologies are observed (Adamcik and Mezzenga 2018) (**B**) In pathological condition, misfolded monomeric α-Syn forms either "on-pathway" or "off-pathway" oligomers, protofibrils and finally mature fibrils. These mature fibrils constitute the inclusion bodies, such as Lewy bodies and Lewy neurites in Synucleinopathies (Mochizuki, Choong et al. 2018) (**C**) In pathological condition, microtubule associated tau protein detached from the microtubules, thus destabilizing the latter. The detached tau starts aggregating forming different stages of aggregates including oligomers, paired helical filaments and finally mature fibrils. These mature fibrils constitute neurofibrillary tangles (Kumar, Kumar et al. 2018).

Misfolded protein aggregates are also known as "amyloids", a term used to describe deposits of Congo red stain-positive large aggregates. In addition to neurodegenerative diseases, amyloid deposits have also been reported in other proteinopathies including, type 2 diabetes, some forms of emphysema, and cystic fibrosis (Mc Laughlin, Crotty et al. 2006, Hartl 2017) with severe social impact. In proteinopathies, a physiological protein loses its

normal function and instead gains a toxic function as it aggregates and accumulates in the cell (Winklhofer, Tatzelt et al. 2008). Fibrils are the building blocks for the large inclusions that are pathological hallmarks of several neurodegenerative diseases. As evidenced by positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), such protein aggregates appear much earlier than the clinical symptoms of the diseases are manifested (Quigley, Colloby et al. 2011, Johnson, Fox et al. 2012, Gomperts, Locascio et al. 2016). During the protein aggregation process, intermediate aggregates such as oligomers, annular oligomers that morphologically appear as 'ring-shaped', protofibrils are formed prior to mature fibril formation (Ross and Poirier 2004, Ross and Poirier 2005). These intermediary aggregates display higher toxic properties at variable degrees compared to the mature fibrils.

Formation of Toxic Amyloid Oligomers

A paradigm shift in neurodegeneration research was supported by the evidence that soluble oligomers or pre-fibrillar oligomers are the actual toxic species which appear intermediately prior to fibril or large inclusions formation (Haass and Selkoe 2007, Stefani 2010, Uversky 2010). Studies have demonstrated that pre-fibrillar oligomeric species of α-Syn are responsible for the disease pathogenesis, but not its fibrillar aggregates (Ingelsson 2016). Oligomers of several amyloidogenic proteins such as α-Syn, tau, amyloid β, huntingtin (Htt), and the prion protein are shown to be associated with multiple neurodegenerative diseases (Glabe 2006, Jucker and Walker 2013). Majority of the amyloid oligomers are structurally variable. They have been classified as either "on-pathway" or "off-pathway" oligomers. On-pathway oligomers necessarily lead to the

formation of fibrils, whereas, off-pathway oligomers can be arrested in their oligomeric state, thus causing enhanced toxicity (Bemporad and Chiti 2012).

α-Syn oligomers

According to the α-Syn cascade hypothesis, aggregates of this protein are formed long before the clinical symptoms are manifested (Ingelsson 2016). Even so, it remains elusive as to what initiates the aggregation of α-Syn into LBs or when and in which brain regions they are first formed. Aggregation of α-Syn is influenced by chemical variabilities such as changes in pH and salt concentrations (Buell, Galvagnion et al. 2014).

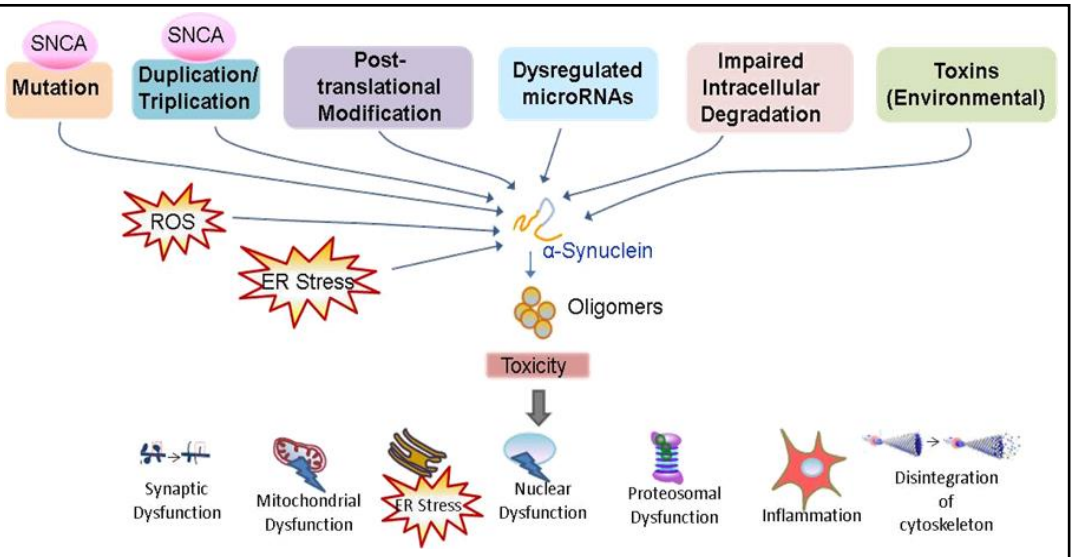

Figure 1. 5 Potential mechanisms for α-Syn oligomer formation and their toxicities. Mutations and multiplications in SNCA gene coding for α-Syn, post-translational modifications, dysregulated microRNAs and environmental toxins may lead to the formation of toxic α-Syn oligomers. Reactive oxygen species (ROS) or stress from endoplasmic reticulum (ER stress) can also lead to oligomers formation. These oligomeric assemblies are shown to disrupt multiple cellular functions including synaptic, nuclear and proteasomal functions. They are also shown to cause toxicity to mitochondria and endoplasmic reticulum in a feedback mechanism. Other dysfunctions include cytoskeleton disintegration and inflammation.

Accumulating evidence suggests that α-Syn aggregation specifically the formation of oligomers is a significant event driving neuronal degeneration in synucleinopathies

impairing multiple cellular pathways. Although the exact mechanism of how α-Syn causes neurodegeneration is still unclear, several studies have demonstrated its role in disrupting nuclear, mitochondrial and synaptic functions (Ingelsson 2016) (**Figure 1.5**). The potential toxic role of α-Syn oligomers in varied cellular functions is commonly described as "α-Syn oligomers hypothesis" (Ono 2017). Extracellularly applied recombinant α-Syn oligomers were shown to cause cell death and impair synaptic plasticity and cognition via altering Ca^{2+} level and inducing calcineurin level (Martin, Neugebauer et al. 2012). Oligomeric α-Syn has been demonstrated to cause endoplasmic reticulum (ER) stress in synucleinopathies (Villar-Pique, Lopes da Fonseca et al. 2016). Oligomeric α-Syn has been demonstrated to cause endoplasmic reticulum (ER) stress in synucleinopathies (Villar-Pique, Lopes da Fonseca et al. 2016). Moreover, these toxic species also cause microtubule disintegration (Prots, Veber et al. 2017). Oligomers of variable sizes were shown to be present in the brain tissues of synucleinopathy patients. Moreover, α-Syn oligomers were also detected in the biological fluids such as, cerebrospinal fluid (CSF) and blood plasma of PD and DLB patients (El-Agnaf, Salem et al. 2006, Noguchi-Shinohara, Tokuda et al. 2009, Tokuda, Qureshi et al. 2010, Hansson, Hall et al. 2014). Accumulation of toxic α-Syn oligomers have been shown in the lumen of endoplasmic reticulum of transgenic animal model expressing mutant A53T α-Syn. Treating these animals with an anti-ER stress molecule, Salubrinal, reduced accumulation of toxic oligomers in the ER and delayed disease onset (Colla, Jensen et al. 2012). Environmental and genetic risk factors have been shown to play roles in oligomers mediated pathogenesis (Ingelsson 2016).

Despite the knowledge of neurotoxic properties of oligomers, mechanisms that drive misfolding and aggregation of pathogenic proteins giving rise to cytotoxic

conformations, are poorly understood and under investigation. To this end, in vitro oligomerized α-Syn assemblies have provided an invaluable approach. α-Syn oligomers with differences in morphology, have been reported to occur, which can be visualized and analyzed by size exclusion chromatography (SEC), atomic force microscopy (AFM), electron microscopy (EM) and cryo-electron microscopy (Cryo-EM) (Waxman and Giasson 2009). However, spherical α-Syn oligomers of 2-6 nm diameter are reported to be a highly neurotoxic species in primary cortical neurons, causing cell loss and abnormal calcium currents (Danzer, Haasen et al. 2007). Multiple laboratories, including ours, have generated well-characterized in vitro oligomers from recombinant α-Syn protein (Danzer, Haasen et al. 2007, Lasagna-Reeves, Castillo-Carranza et al. 2010). Utilizing such in vitro oligomers, various techniques and reagents are being developed. These resources enable the investigation of the in vivo occurring α-Syn oligomers , as well as the mechanisms of their adverse biological roles (Danzer, Haasen et al. 2007, Martin, Neugebauer et al. 2012). As mentioned above, α-Syn is highly susceptible to interaction with biological membranes through its N-terminus. Oligomeric aggregates of α-Syn are shown to disrupt lipid bilayer, impairing the cell membrane integrity (Fusco, Chen et al. 2017). Knowledge obtained from in vitro oligomers can be extended to develop therapeutic interventions for synucleinopathies.

Animal Models for Synucleinopathies

Among all the point mutations, the transgenic mouse model overexpressing mutant A53T α-Syn has been most widely studied (Alam, Bousset et al. 2019). In this animal model, oligomeric α-Syn were shown to accumulate in the mitochondrial membrane and impair complex I function (Chinta, Mallajosyula et al. 2010, Colla, Jensen et al. 2012).

Treating A53T transgenic mice with an anti-ER stress molecule, Salubrinal, reduced the accumulation of toxic α-Syn oligomers in the ER and delayed the onset of disease (Colla, Jensen et al. 2012). Transgenic mouse model expressing A30 mutant α-Syn protein develops LB pathology and behavioral impairment (Kahle, Neumann et al. 2000). In this mouse model, an increasing level of protofibrillar α-Syn has been reported with increase of age (Lindstrom, Fagerqvist et al. 2014). Another point mutation variant of α-Syn, E46K, has been associated with familial form of PD and DLB as well. This mutation was reported in a family from the Basque Country with autosomal dominant Parkinsonism who fulfilled the clinical criteria and pathological features for DLB patients (Zarranz, Alegre et al. 2004).

Tau oligomers

For a long period of time, NFTs have been considered as the main pathological hallmark for tauopathies. This consideration was ruled out by the observation that NFT-containing neurons were able to survive for years in both human and mice brains (Morsch, Simon et al. 1999, de Calignon, Fox et al. 2010). Recent studies suggest that intermediate forms of tau aggregation between monomeric form and NFTs, called as tau oligomers are the true neurotoxic species. Hence, these oligomeric assemblies are also being considered as the best therapeutic targets (Spires, Orne et al. 2006, Berger, Roder et al. 2007, Maeda, Sahara et al. 2007, Cowan, Bossing et al. 2010, Kopeikina, Carlson et al. 2011, Sahara 2013). Multiple laboratories, including ours have demonstrated that tau oligomers impair memory, cause synaptic dysfunction and cellular toxicity in AD (**Figure 1.6**) (Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Fa, Puzzo et al. 2016, Puzzo, Piacentini et al. 2017). In our laboratory, we were also able to isolate and characterize tau oligomers from PSP brain tissues that exhibited seeding of

monomeric tau protein, suggesting that tau oligomers from pure tauopathy might have a mechanism common to other disease like AD (Gerson, Sengupta et al. 2014). It has been

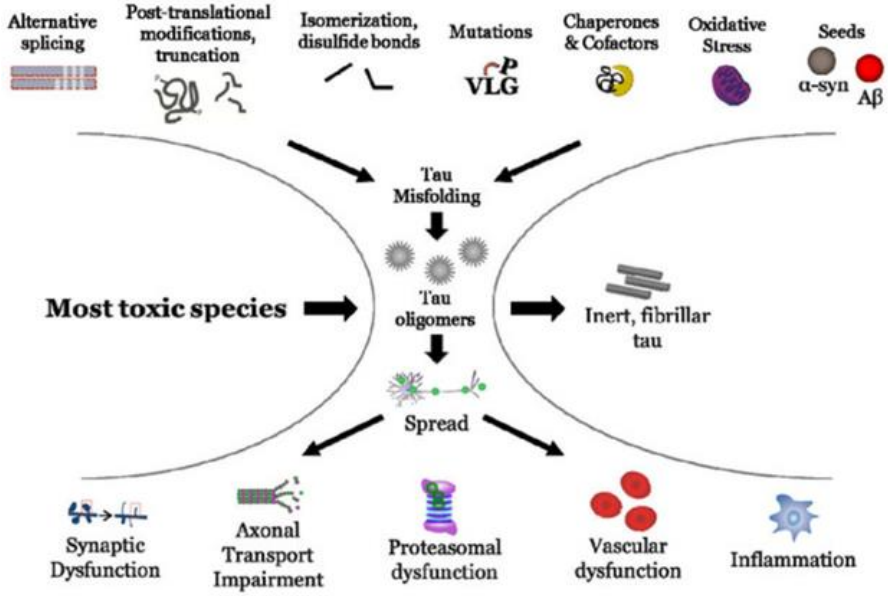

Figure 1. 6 Formation toxic tau oligomers and their role in multiple cellular processes. Under the influence of several factors such as alternative splicing, post-translational modifications, mutations, chaperones and other cofactors, oxidative stress and even other amyloidogenic protein aggregates can lead to form tau oligomers from misfolded tau. These tau oligomers propagate and impair several cellular functions at different organelles such as at synapses, axons, proteasome, vasculature and even cause inflammation (Gerson, Mudher et al. 2016)

shown that tau oligomers derived from diseased brain tissues are highly toxic species with high seeding propensity. Such brain-derived tau oligomers when injected into wild type mice, they spread from site of injection to distant brain regions, causing behavioral deficits in the animals, while fibrillar tau were not able to propagate or induce such toxic effects (Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Wu, Herman et al. 2013). These observations indicate that pathological tau oligomers might propagate in a manner similar to that of prion proteins, known as prion-like mechanism (Gerson and Kayed 2013).

AMYLOID POLYMORPHISMS AND PRION-LIKE PROPERTIES

Amyloid Polymorphisms

Fibrillary structures of amyloids are the building blocks of large inclusions that are found as hallmark aggregates in the autopsied brain tissues for many neurodegenerative diseases. Unlike oligomers which have β-sheet conformation, amyloid fibrils primarily possess cross-β-sheet conformation in which the β-strands are transversely wrapped around the main fiber axis, thereby forming an intermolecular network of hydrogen bonds (Riek and Eisenberg 2016). Studies performed in the last one and half decades led to the discovery of a remarkable characteristic feature of amyloid fibrils, referred to as "polymorphism". Polymorphism refers to the variabilities present at the morphological and structural levels of amyloid fibrils which surprisingly exist among amyloid fibrils composed of a single protein. Such fibrillary polymorphism has been extensively studied in case of amyloid β protein. Apart from amyloid-β protein, α-Syn has also been shown to form structurally distinct fibrillar structures in vitro, indicating their polymorphic nature (Tycko 2015, Li, Zhao et al. 2018). Studies by Eisenberg and co-workers have shown that there are some segments in the polypeptide chain of amyloid β protein, which confer the amyloid structure (Thompson, Sievers et al. 2006). Using cryo-EM, it has been shown that Aβ40 can form 12 different polymorphic fibrillary structures under the same condition (Meinhardt, Sachse et al. 2009). The occurrence of conformationally distinct amyloid-β deposits has been demonstrated in AD brain tissues by using luminescent conjugated polythiophene probes (Nilsson, Aslund et al. 2007). Two different structures of amyloid-β fibrils were observed in the brain tissues of two AD patients, indicating in vivo occurring polymorphisms of amyloid-β (Lu, Qiang et al. 2013).

The Prion Strain Phenomenon

A group of highly infectious neurodegenerative diseases, collectively known as transmissible spongiform encephalopathy (TSE) or prion diseases, is caused by the infectious protein, prion (PrP) (Collinge, Sidle et al. 1996). Although PrP is the sole pathogenic protein for prion diseases, it is shown that the cellular form of this protein (PrP^{c}) is not the toxic entity. Instead, its pathogenic form, which was discovered in Scrapie disease (PrP^{sc}) is the actual causative agent in prion diseases (Peretz, Scott et al. 2001). Animals affected with prion diseases exhibited different phenotypes. One of the most baffling phenomena in the field of prion biology is that animals infected with the same pathogenic infectious agent displayed different clinical phenotypes (Bessen and Marsh 1992, Bartz, Bessen et al. 2000, Klimova, Makarava et al. 2015). Moreover, when such infectious protein aggregates were isolated from brain tissues of prion disease and inoculated in the identical hosts, they induced distinct phenotypes that were specific to the prion diseases (Morales 2017). Prior studies have revealed the association between variable phenotypes observed in the same prion disease and the formation of different aggregates by the same prion protein. Such different aggregates are termed as strains (Stein and True 2014, Morales 2017). The nomenclature of these variable prion strains, although devoid of any genetic material, were in accordance with other infectious agents (such as microbes). It was observed that each prion strain caused different phenotypes (Baylis and Goldmann 2004). The cellular and pathogenic forms of prion can readily be distinguished in vitro based on their physical properties, such as denaturation with detergents, proteolytic digestion and their secondary structures (Morales, Abid et al. 2007). The in vivo characteristics of prion diseases include incubation period, which is defined by the period from inoculation to

clinical manifestations, damage to the brain regions identified by histopathological analyses and clinical symptoms. These characteristics are also used to distinguish between prion strains (Bruce 1993, Morales, Abid et al. 2007). The basis of such strain formation is described as the ability of PrP protein to adopt different conformational states, giving rise to different aggregates with variable properties (Eisenberg and Jucker 2012). An important characteristic of prion strains is that they can be serially passaged within the same species or between different susceptible species (Morales 2017). In experimental mouse models, more than 20 distinct prion strains were isolated which were phenotypically different enabling the study of prion strain phenomenon (Bruce 1993). Another aspect of prion disease is that several strains can be found in different brain regions of the same patient. Such bewildering observation was assumed to be the underlying reason for individual variability shown in clinical manifestations (Schoch, Seeger et al. 2006).

Prion-like Amyloids

It is a now widely accepted that the spreading of pathogenic protein aggregates is a necessary event in the disease progression. To this end, studies have also been directed to dissect out the mechanism by which amyloidogenic protein aggregates spread from cell to cell. Walker Lc et al, has suggested a templating mechanism of misfolded protein in which once a small amount of aggregate is formed, it serves as “seeds” for further aggregation. Such seeds can recruit more nascent protein by templating their conformation to the newly added proteins (Walker Lc 2013). Accumulating experimental evidence suggests that many neurodegenerative diseases share the phenomenon of prion disease, where the pathogenic protein aggregates seed for aggregation in a self-replicating manner in spite of the lack of infectivity (Polymenidou and Cleveland 2011). Thus, amyloidogenic protein aggregates,

which can seed into structurally different polymorphs and propagate in a prion-like manner, are considered as prion-like amyloids. Increasing evidence is suggesting that prion-like proteins such as Aβ, α-Syn, tau and TDP-43 share a common paradigm of replication as well (**Figure 1.7**).

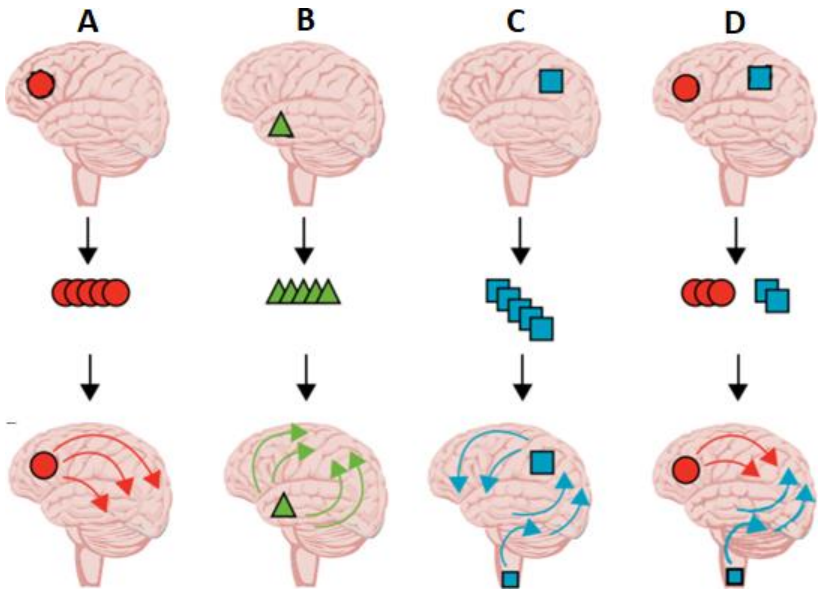

Figure 1. 7 Schematic representation of amyloid strains and their propagation. Multiple strains (A, B, C and D) of amyloid protein as depicted by different shapes and colors can occur in different regions of brain in different diseases, which can be isolated and characterized in vitro. Such strains may have different patterns of propagation in anatomically connected brain region with different rates of spreading as shown by arrows. Strains of single protein with diverse spreading characteristics may account for variable pathological and clinical features. This model can be applied to any amyloidogenic protein strains such as α-Syn, tau many others. Modified from Diamond et al., 2017 (Stopschinski and Diamond 2017)

Synucleinopathies include diverse diseases such as PD, DLB, MSA and AD. In a study, autopsied brain tissue of a PD patient receiving transplantation of grafted embryonic nigral neurons showed LB pathology (Kordower, Chu et al. 2008) . In another study, two PD patients receiving grafts underwent autopsies after 11 and 14 years of the grafting. In both the cases, some of the grafted neurons were LB-positive (Li, Englund et al. 2008).

These studies suggested that α-Syn aggregates propagate in anatomically connected neural networks. Similarly, intracerebral injections of brain homogenates from patients with different tauopathies, induced tau pathology in wild type mice close to the injection site, which later on spread in anatomically connected distant brain regions (Clavaguera, Akatsu et al. 2013). Moreover, when distinct fibrillar tau aggregates isolated from cell cultures were injected into mice, they induced unique pathological features, suggesting the propagation of the tau aggregate strains (Sanders, Kaufman et al. 2014).

Characterization of Amyloid Strains

Prion proteins are usually identified by employing widely used techniques based on their biological and biochemical properties. The biological properties include i) the incubation period of the protein from its infection to clinical manifestation and ii) the region-specific damage caused by the aggregation of different strains. Biochemical properties identifying the majority of prion strains include their glycosylation profile, resistance to protease enzyme, electrophoretic mobility, and stability to sedimentation (Morales 2017). These discoveries have opened up a new era of research unravelling the plausible occurrence of different conformations of other amyloidogenic proteins such as Aβ and α-Syn (Stohr, Watts et al. 2012, Watts, Condello et al. 2014). However, whether these strains from other amyloidogenic proteins are infectious or not remains debatable. The established standard protocols for prion strains are also being applied for isolation and characterization of α-Syn , Aβ and tau aggregate strains. Multiple laboratories including ours have developed protocols, optimized techniques for isolating and characterizing amyloid strains from different disease cases and demonstrated their seeding properties

(Sanders, Kaufman et al. 2014, Gerson, Sengupta et al. 2017, Kaufman, Thomas et al. 2017, Sengupta, Carretero-Murillo et al. 2018).

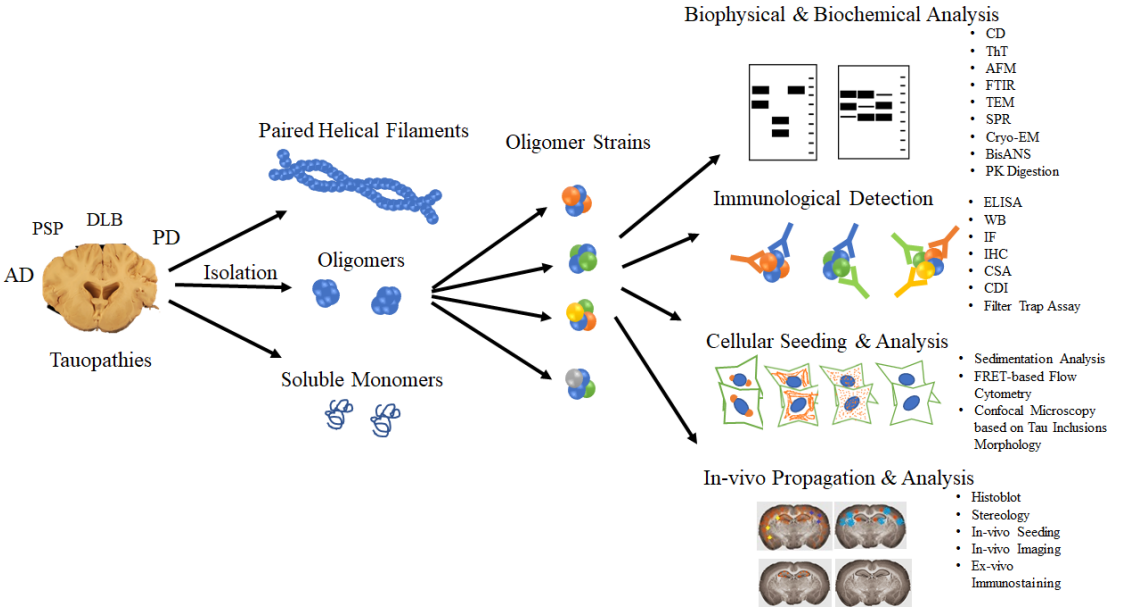

Figure 1. 8 Schematic representation of isolation and characterization of tau oligomeric strains (Sengupta, Carretero-Murillo et al. 2018).
These methods can be applied to strains of any amyloid protein.

These techniques include an array of several complementary methods of biochemical, biophysical, immunological, cellular and in vivo characterization of amyloid strains (**Figure 1.8**). And hence, these techniques or methods can be applied for establishing strains of any amyloid protein.

INTERACTIONS AND POTENTIAL SYNERGY BETWEEN PROTEIN AGGREGATES IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases frequently display multiple or mixed protein pathologies, which often interact and modulate the normal functioning as well as the downstream effects of these proteins. Although amyloidogenic proteins might follow unique cascades of pathological events, majority of them share certain common mechanisms for toxicity. Co-occurrence of multiple protein pathologies represent a wide

range of neurodegenerative disorders where comorbidity and overlap between the diseases are frequently observed (Moussaud, Jones et al. 2014). In addition to LB, senile plaques of Aβ, NFTs and neuropil threads composed of tau were observed in the cerebral cortex of PD brain tissues (Braak and Braak 1990). Senile plaques and NFTs are often found together in DLB cases (Jacobson, Morshed et al. 2014). Thus, there is a considerable overlap between α-Syn, tau and Aβ protein pathologies in synucleinopathies, suggesting their potential synergistic effects on disease pathogenesis. Oligomeric assemblies of Aβ were shown to colocalize with other amyloidogenic proteins such as α-Syn, PrP and TAR DNA-binding protein 43 (TDP-43) (Guerrero-Munoz, Castillo-Carranza et al. 2014). In animal and cell models, Aβ was shown to be a contributor for tau aggregation. In P301L mice, a transgenic mouse model of tauopathy, fibrillar Aβ42 was shown to induce NFT formation (Gotz, Chen et al. 2001). In a cellular model overexpressing human tau, pre-aggregated Aβ42 was shown to cause paired helical filament (PHF) formation (Ferrari, Hoerndli et al. 2003, Pennanen and Gotz 2005). In APP/PS1 transgenic animal model, α-Syn was shown to inhibit Aβ plaque formation (Bachhuber, Katzmarski et al. 2015).

In recent years, the role of RNA-binding proteins (RBPs) and their aggregates are being studied in neurodegenerative diseases. It has been shown that tau interacts with T cell intracellular antigen 1 (TIA1), which is an RBP and an important component of stress granules. Such interaction regulates tau aggregation and stress granule formation (Vanderweyde, Apicco et al. 2016). Recently we have demonstrated the colocalization of tau oligomers with Musashi proteins (MSI1 and MSI2), a family of RBPs in the postmortem AD brain tissues. Moreover, we have observed that tau and MSI mediate the

aggregation of each other in the nucleus and cytoplasm of a cellular model, indicating their synergistic effects (Sengupta, Montalbano et al. 2018, Montalbano, McAllen et al. 2019).

INTERACTIONS BETWEEN ALPHA-SYN AND TAU IN SYNUCLEINOPATHIES

Many neurodegenerative diseases share common features in terms of their late-life clinical manifestations as well as protein aggregation, loss of synapse and cell death as revealed by the postmortem histopathological analyses (Gadad, Britton et al. 2011). Several pathogenic proteins interact in neurodegenerative diseases, thus regulating the normal functions of each other (Clinton, Blurton-Jones et al. 2010). Even though the major pathological hallmarks of synucleinopathies are α-Syn aggregates, pathology of abnormal tau aggregates, NFTs, have long been studied in DLB, PD and MSA diseases (Jellinger 2009, Moussaud, Jones et al. 2014). Co-morbidity of AD and DLB is a major and common problem, where almost 85% of DLB cases are shown with abundant amyloid plaques, one of the pathological hallmarks of AD (Gomperts, Locascio et al. 2016). Moreover, LBs in PD and DLB cases were shown to contain hyperphosphorylated tau (Arima, Hirai et al. 1999, Iseki, Takayama et al. 2002). Similarly, aggregated α-Syn pathology is also demonstrated in AD pathology (Larson, Sherman et al. 2012). DLB and AD share many clinical and histopathological features, making the diagnosis of DLB a challenge. At autopsy, more than half of AD patients showed LBs (Hamilton 2000). Several neurodegenerative disorders that are pathologically characterized by α-Syn protein aggregates, are also associated with aggregates of the microtubule-associated protein tau into NFTs such as PD (Spillantini, Schmidt et al. 1997, Ishizawa, Mattila et al. 2003) , PDD, DLB and MSA (Iseki, Takayama et al. 2002, Lei, Ayton et al. 2010, Nagaishi, Yokoo

et al. 2011, Colom-Cadena, Gelpi et al. 2013). It has been postulated that both α-Syn and tau proteins possess synergistic effects on each other, resulting in the fibrillar amyloid structures formation (Giasson, Forman et al. 2003, Guo, Covell et al. 2013). Exogenously added fibrils of recombinant tau were shown to seed for α-Syn fibrilization in different cell models (Badiola, de Oliveira et al. 2011). The seeding ability of pre-formed fibrillar α-Syn to form cytosolic tau inclusions encompassing almost the entire cytosol, has been shown in human embryonic kidney cell line, QBI293 cells (Waxman and Giasson 2011). These studies strongly argue about the crosstalk between α-Syn and tau.

Transgenic mouse model overexpressing human α-Syn mutation, A53T, showed abundant tau pathology in several brain regions (Giasson, Forman et al. 2003), whereas in another study, transgenic mouse model overexpressing human wild type (WT) α-Syn, exhibited spontaneous age-dependent tau pathology (Haggerty, Credle et al. 2011). In vitro, recombinant oligomeric assemblies of α-Syn were shown to induce tau aggregation, specifically oligomer formation, indicating the cross-seeding mechanism between these two proteins (Lasagna-Reeves, Castillo-Carranza et al. 2010). Passive immunotherapy against tau oligomers shows beneficial effects by preventing cognitive and motor deficits as well as brain protein pathology in transgenic A53T animal model (Gerson, Farmer et al. 2018). More recently, the role of tau has also been demonstrated in mediating impairment of hippocampal neurotransmission and memory deficits in the same A53T transgenic animal model, suggesting a tau-dependent mechanism of α-Syn pathology (Singh, Covelo et al. 2019). These studies strongly argue the importance of functional crosstalk between α-Syn and tau. Studies have shown that α-Syn pathology is an upstream event of tau pathology (Giasson, Forman et al. 2003, Waxman and Giasson 2011), which raises the

question on the type of the interaction between these two proteins. One suggested interaction is an ***indirect interaction*** (**Figure 1.9)**. Several mitochondria-related genes such as Leucine-rich repeat kinase 2 (LRRK2), Parkin and PTEN-induced kinase-1 (PINK1) are strongly associated with PD pathogenesis (Klein and Westenberger 2012). A direct

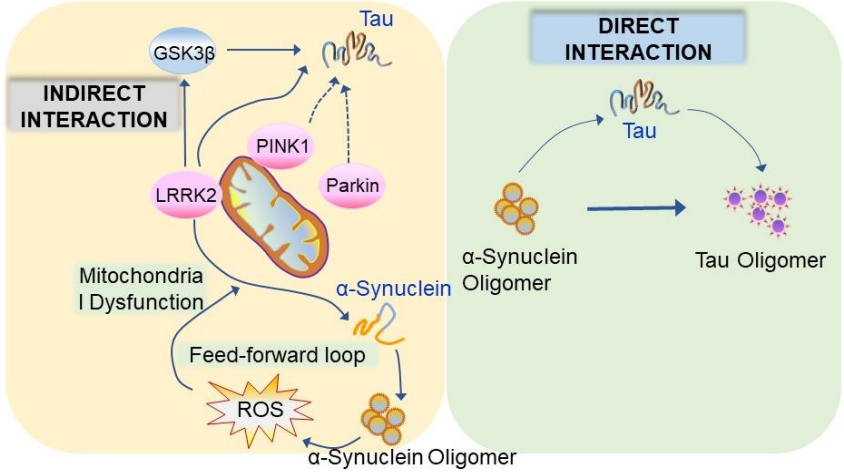

Figure 1. 9 Schematic representation of interaction between α-Syn and tau proteins. Studies have demonstrated an indirect interaction via various kinases of mitochondria and other interacting proteins of tau. A few experimental studies indicate a direct interaction between these two proteins as well.

interaction between LRRK2 and tau protein and its other interacting proteins such as Glycogen synthase kinase 3β (GSK3β) has been demonstrated (Kawakami, Yabata et al. 2012, Kawakami, Shimada et al. 2014, Guerreiro, Gerhardt et al. 2016). These studies indicate an indirect interaction between α-Syn and tau protein in disease pathogenesis. Alternatively, a ***direct interaction*** between α-Syn and tau has also been suggested (**Figure 1.9)**. However, there is not enough investigation done on the direct interaction between α-Syn and tau. A very early in vitro study has shown that α-Syn directly interacts with the MTBR of tau protein, modulating the phosphorylation state at least at two serine residues of the latter (Jensen, Hager et al. 1999). We have previously reported that in vitro α-Syn oligomers are capable of inducing the aggregation of monomeric tau and forming tau

oligomers, thus indicating a cross-seeding process (Lasagna-Reeves, Castillo-Carranza et al. 2010). Our laboratory was the first to demonstrate that, in addition to α-Syn oligomers, tau oligomers are also present in the postmortem sbrain tissues of PD and DLB patients (Sengupta, Guerrero-Munoz et al. 2015). In our recent study, we have demonstrated that the tau aggregates cross-seeded with pre-formed α-Syn oligomers evade larger aggregate or fibril formation, thus, prolonging their oligomeric state compared to the self-aggregated tau. The cross-seeded tau aggregates were also shown to be more toxic than the self-aggregated tau. These studies demonstrate the combined deleterious effects of α-Syn and tau, suggesting a toxic mechanism of interaction.

RATIONALE

The formation of α-Syn oligomers is a critical event in the pathogenesis of synucleinopathies such as DLB and PD. Similarly, oligomeric tau is considered as a significant player initiating neurotoxicity in tauopathies, including AD. There is a significant overlap between multiple neurodegenerative diseases, both in terms of their clinical manifestations and pathological protein aggregates. Co-morbidity of DLB and AD is a common phenomenon and pose a major problem in their accurate diagnosis as these diseases share many clinical and histopathological features. Accumulating evidence suggests the coexistence of α-Syn and tau aggregates in multiple diseases. However, some critical points remain still to be answered such as, i) what is the role of α-Syn in tau aggregation, and ii) what is the toxic synergy between α-Syn and tau. Understanding the potential interaction between the two proteins, specifically at their toxic oligomeric states,

and the downstream effects of such interaction is crucial. The hypothesis tested in this thesis is that, α-Syn forms discrete oligomeric strains and that these oligomeric strains interact with tau, forming distinctive aggregated tau strains. The findings from this work demonstrates strain-specific interaction between α-Syn and tau, opening up new avenues for neuroprotective intervention strategies in synucleinopathies by targeting multiple candidate molecules.

A part of 'Characterization of Amyloid Strains' section has been taken from "Sengupta U, Carretero-Murillo, M, Kayed R (2015). Preparation and Characterization of Tau Oligomer Strains. Methods Mol Biol. 1779:113-146. doi: 10.1007/978-1-4939-7816-8_9"

CHAPTER 2

POLYMORPHIC ALPHA-SYNUCLEIN STRAINS MODIFIED BY DOPAMINE AND DOCOSAHEXAENOIC ACID

ABSTRACT

The pathological hallmark of synucleinopathies, including Parkinson's disease (PD), is the aggregation of α-Synuclein (α-Syn) protein. Even so, tau protein pathology is abundantly found in these diseases. Both α-Syn and tau can exist as polymorphic aggregates, and this phenomenon has been widely studied, mostly in their fibrillar assemblies. We have previously discovered that in addition to α-Syn oligomers, oligomeric tau is also present in the brain tissues of patients with PD and dementia with Lewy bodies (DLB). While the pathophysiology of both α-Syn and tau oligomers has been investigated in different neurodegenerative diseases, the functional crosstalk between these toxic oligomers has not been scrupulously studied. Here, we have explored the structural and functional diversity of distinct α-Syn oligomers, prepared by modifying the protein with dopamine (DA) and docosahexaenoic acid (DHA). The two α-Syn oligomers differed in aggregate size, conformation, sensitivity to proteinase K digestion, tryptic digestion and toxicity, suggesting them as distinct α-Syn oligomeric strains. We examined the mechanisms by which they are internalized in primary neurons, as well as their seeding propensity in inducing cytosolic α-Syn aggregation.

BACKGROUND

Synucleinopathies consist of multiple neurodegenerative diseases, among which Parkinson's disease (PD) is one of the three major Synucleinopathies (Spillantini, Crowther et al. 1998, Spillantini and Goedert 2000, Goedert, Jakes et al. 2017). Aggregates of α-Synuclein (α-Syn) are the primary causative protein aggregates that are established as the pathological hallmarks for Synucleinopathies, including PD. α-Syn is primarily a presynaptic protein and is abundantly expressed in the brain (Spillantini, Schmidt et al. 1997). Several studies have shown that amyloid oligomers can be formed by different mechanisms and can even exert their toxic effects in different ways (Gerson, Mudher et al. 2016). It has been highly debatable subject as to which forms of aggregated amyloids exhibit strongest seeding potency and maximum toxicity. However, an increasingly accepted hypothesis is that oligomeric forms of amyloidogenic proteins such as α-Syn, tau, amyloid-β and many others are the most toxic intermediates causing impairment in several cellular processes (Conway, Lee et al. 2000, Kayed, Head et al. 2003, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Kayed and Lasagna-Reeves 2013, Chen, Drakulic et al. 2015, Sengupta, Nilson et al. 2016, Bengoa-Vergniory, Roberts et al. 2017, Nichols, St-Pierre et al. 2019). In addition to α-Syn protein pathology, synucleinopathies also exhibit abundant tau pathology in the form of neurofibrillary tangles (NFTs), which has also been long studied in PD (Braak and Braak 1990, Haggerty, Credle et al. 2011, Zhang, Gao et al. 2018) and dementia with Lewy bodies (DLB) (Popescu, Lippa et al. 2004, Colom-Cadena, Gelpi et al. 2013). Tau is a microtubule binding protein with six alternatively spliced isoforms (Ballatore, Lee et al. 2007). Accumulating evidence suggest that intermediary tau oligomers are the true neurotoxic species. Due to their toxic effects

impairing several cellular processes, they are considered as the therapeutic targets (Maeda, Sahara et al. 2006, Spires, Orne et al. 2006, Lasagna-Reeves, Castillo-Carranza et al. 2011, Shafiei, Guerrero-Munoz et al. 2017). Our laboratory has extensively studied tau oligomers in many neurodegenerative diseases including Alzheimer's disease (AD) (Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012), progressive supranuclear palsy (PSP) (Gerson, Sengupta et al. 2014), traumatic brain injury (TBI) (Hawkins, Krishnamurthy et al. 2013) and Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Castillo-Carranza, Nilson et al. 2017), as well as in synucleinopathies such as, PD and DLB cases (Sengupta, Guerrero-Munoz et al. 2015). We have previously showed that in addition to the α-Syn oligomers, oligomeric tau is also present in PD and DLB brain tissues. Moreover, we observed that these two oligomeric species co-existed in the same aggregate (Sengupta, Guerrero-Munoz et al. 2015). In our recent study, we observed that complexes of oligomeric α-Syn and tau isolated from PD brain tissue were more potent causing behavioral impairment in Htau animals (Castillo-Carranza, Guerrero-Munoz et al. 2018). The presence of overlapping protein pathologies in several neurodegenerative diseases, including PD (Moussaud, Jones et al. 2014, Irwin and Hurtig 2018), support the phenomenon of protein cross-seeding. Previously, we have demonstrated that α-Syn can cause tau aggregation in vitro (Lasagna-Reeves, Castillo-Carranza et al. 2010) and such cross-seeding can result in a more toxic form of tau oligomers (Castillo-Carranza, Guerrero-Munoz et al. 2018, Sengupta and Kayed 2019). Tau and α-Syn are shown to induce fibrillization of each other in vitro (Giasson, Forman et al. 2003).

Amyloidogenic proteins such as amyloid-β and α-Syn can form structurally distinct fibrillar structures in vitro, indicating their polymorphic nature (Tycko 2015, Li, Zhao et al. 2018). The occurrence of conformationally distinct amyloid-β deposits has been demonstrated in AD brain tissue by using luminescent conjugated polythiophene probes (Nilsson, Aslund et al. 2007). Amyloid-β fibrils extracted from the brain tissues of two AD patients showed differences in their molecular structures, indicating polymorphisms of endogenously occurring amyloid-β (Lu, Qiang et al. 2013). Studies have indicated that protein aggregates spread from one brain region to another in a "prion-like" manner (Brundin, Melki et al. 2010, Goedert, Clavaguera et al. 2010, Prusiner 2012). The discovery that a single protein such as tau can form different inclusions in different neurodegenerative diseases, collectively known as tauopathies, suggests the presence of "amyloid strains" with distinct properties (Prusiner 2012, Clavaguera, Akatsu et al. 2013). To this end, the occurrence of fibrillar tau strains have also been demonstrated in different tauopathies (Sanders, Kaufman et al. 2014). Also, fibrillar α-Syn isolated from brain tissues of one PD and one multiple system atrophy (MSA) patient showed variable strain characteristics (Prusiner, Woerman et al. 2015).

Different conditions and cofactors have been shown to cause different conformational and aggregation states of α-Syn (Breydo, Wu et al. 2012). Conway et al., showed that the interaction between DA and α-Syn by stabilizing the latter as adducts, resulting in the formation of α-Syn protofibrils (Conway, Rochet et al. 2001). Later it was demonstrated that in addition to the disaggregation of existing α-Syn fibrils, DA rather inhibits further fibrilization of α-Syn (Li, Zhu et al. 2004). Studies have shown that DA stabilizes α-Syn by forming SDS-resistant, stable soluble oligomers that appear small and

irregular under electron microscope (Cappai, Leck et al. 2005, Lee, Baek et al. 2011). Interaction of α-Syn with lipid membranes is a well-known phenomenon. Studies suggest that increased levels of polyunsaturated fatty acids (PUFAs) are associated with oligomerization of α-Syn (Sharon, Bar-Joseph et al. 2003, Assayag, Yakunin et al. 2007). Docosahexaenoic acid (DHA), a PUFA, is abundantly present in the brain. It is reported that α-Syn regulates fatty acid metabolism in the brain, and moreover, it binds with cerebral PUFAs, such as DHA (De Franceschi, Frare et al. 2011, De Franceschi, Fecchio et al. 2017). The ability of DHA to form α-Syn oligomers in vitro has also been demonstrated (Fecchio, De Franceschi et al. 2013, De Franceschi, Fecchio et al. 2017).

As majority of the amyloid strains' studies are performed on their fibrillar, there is limited knowledge about polymorphic strains of α-Syn and tau in their toxic oligomeric states and the effect of their interaction is still under investigation. Here, we show that α-Syn can form entirely two distinct oligomeric strains by two biological inducers, DA and DHA. To the best of our knowledge, this is the first study where two particularly disease-relevant conditions have been used to establish α-Syn oligomeric strains by thoroughly characterizing and comparing their biochemical, biophysical and biological properties.

METHODS

Preparation of α-Syn Oligomers

Recombinant human full length α-Syn protein was expressed in *E. coli* and purified. Purified protein was dialyzed overnight against water and lyophilized. Lyophilized α-Syn protein was dissolved in 50% acetonitrile as 1 mg/mL and relyophilized. Oligomers of α-Syn without any modification was prepared following our published protocol (Lasagna-

Reeves, Castillo-Carranza et al. 2010, Sengupta, Guerrero-Munoz et al. 2015). Briefly, an aliquot of thus relyophilized protein was dissolved in 280 µl of hexafluoroisopropanol (HFIP) and allowed to incubate at room temperature (RT) for 10–20 minutes in 2 ml eppendorf tube. Double-distilled H_2O was added to this solution to make the final concentration 0.7 µg/µl. The resulting solution was then stirred with a Teflon-coated micro stir bar at 500 RPM for 48 hours inside the fume hood with a perforated cap with allowing the evaporation of HFIP. This oligomeric preparation was used as control α-Syn oligomers for comparison purpose.

Preparation of DA-Modified α-Syn Oligomers

To prepare DA modified oligomers, we followed a previously published protocol by Lee et al., with modification (Lee, Baek et al. 2011). Dopamine hydrochloride (Sigma, H8502) was dissolved in 20 mM Tris buffer (pH 7.4) and 100 mM NaCl solution to obtain a final concentration of 100 mM DA solution. Relyophilized α-Syn protein was immediately dissolved in the freshly prepared DA solution at 1:20 molar ratio (protein:DA) to obtain a final protein solution of 50 µM α-Syn: 1 mM DA. This solution was incubated at 300 RPM at 37^0C for 110 h. At the end of incubation, the solution was centrifuged at 16,000 g for 10 min and the supernatant was collected.

Preparation of DHA-Modified α-Syn Oligomers

α-Syn oligomers modified by DHA was prepared following published protocol (Fecchio, De Franceschi et al. 2013). Briefly, purified and relyophilized α-Syn protein (0.7 µg/µl) was dissolved in 1 X PBS solution. cis-4,7,10,13,16,19-Docosahexaenoic acid (Sigma,

53171) was added to the α-Syn solution at a molar ratio of 1:50 (protein:DHA) and incubated at 37^0C for 48 h at 500 RPM.

Preparation of fibrils

Fibrils of α-Syn were prepared by following our published method (Ghag, Bhatt et al. 2018). Briefly, recombinant purified α-Syn was dissolved in water with physiological salt concentration and stirred for 6-7 days at 37^0C. Sodium azide was added at 0.01% to the final solution to avoid bacterial contamination.

Western blot Analysis

Three different concentrations of α-Syn oligomer preparations as well fibrillar α-Syn sample were loaded on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were transferred onto nitrocellulose membranes and blocked with 10% nonfat dry milk prepared in 1X TBST buffer at 4°C overnight. Membranes were then probed with primary antibodies, LB509 (1:2000; Abcam, Ab27766) diluted in 5% nonfat dry milk for 1 h at RT. To detect the LB509 signal, HRP-conjugated anti-mouse IgG (1:6000, GE Healthcare) was used. ECL plus (GE Healthcare) signal developing reagent was used to visualize the bands. Densitometric analysis of the bands was performed using ImageJ software (National Institutes of Health).

Size-exclusion Chromatography

All α-Syn oligomers preparations were analyzed using AKTA Explorer system that was fitted with a Superdex 200 Increase 10/300 GL Column. As a mobile phase, degassed deionized water was used with a flowrate of 0.5 mL/min. For calibrating the samples peaks,

a gel filtration standard (Bio-Rad, 51-1901) was used. Sample peaks resolved at 280 nm absorbance were obtained.

Proteolytic Digestion of α-Syn Oligomers by Proteinase K Enzyme

Different oligomer preparations of α-Syn (10-12 µg) were treated with different concentrations of proteinase K enzyme (Sigma) ranging from 1-2 µg/mL in presence of 1 X PBS buffer and incubated at 37^0C for 30 min. At the end of incubation time, 1 X LDS sample buffer (Invitrogen) was added and heated at 95 ^{0}C for 10 min. Samples were immediately transferred onto ice to stop the cleavage reaction followed by loading the digestion products into 4-12% bis-tris precast gels (Invitrogen) for SDS-PAGE gel electrophoresis. Samples with all conditions were run in two sets for electrophoresis. Gels with one set of digested samples were processed for silver staining (Pierce Silver Stain Kit, Thermo Scientific; 24612) to visualize the fragments following manufacturer's instructions. Another set of digested samples were transferred onto nitrocellulose membrane for Western blot analysis immunolabeled with LB509 antibody to visualize the PK-resistant aggregates.

Atomic Force Microscopy

Different oligomer preparations of α-Syn, fibrillar α-Syn were analyzed by AFM using a non-contact tapping method with a Multimode 8 AFM machine (Bruker, Billerica MA). Briefly, 3-4 µl of each sample was added onto a freshly-cleaved mica surface attached to a metallic disc. The samples were allowed to be adsorbed on the mica surface at RT overnight. Mica was then washed with 200 µl of deionized followed by air-drying. Images were taken from 5 different areas on the mica surface.

Bis-ANS and Thioflavin T Fluorescence Assays

Three µl of either α-Syn (0.5 µg/ µl) and 247 µl of 10 µM bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt) prepared in mM glycine-NaOH buffer (pH 7.4) were added to the 96-well clear-bottomed black plates. Each condition was analyzed in triplicates. The fluorescence intensity was measured at 520 nm λ-emission upon 380 nm λ-excitation. For Thioflavin T (ThT) assay, 3 µl of protein (0.5 0.6 µg/ µl) and 247 µl of 20 µM ThT prepared in 50 mM glycine-NaOH buffer (pH 8.5) were added to the wells in triplicates. The fluorescence intensity was read at 490 nm λ-emission upon 440 nm λ-excitation using a plate reader (POLARstar OMEGA; BMG Lab technologies). Each condition for this assay was performed in triplicates.

Circular Dichroism

Circular dichroism spectra of samples were measured in a spectropolarimeter Jasco-720 (JASCO Inc.) equipped with a temperature controller as published earlier (Guerrero-Munoz, Castillo-Carranza et al. 2013). Spectra were recorded at 0.20-nm intervals with a scan speed of 20 nm/min in a quartz cell of 1 mm pathlength. The protein concentration used was 0.1 µg/µl at RT. Spectra were measured in 1 X PBS buffer (pH 7.4) from 195 nm to 250 nm and an average of 3 iterations were recorded for each spectrum. The quartz cell was washed with water and ethanol between every use. Proteins' secondary structures were estimated from CD spectra using K2D3 software, an updated version of K2D2 software (Louis-Jeune, Andrade-Navarro et al. 2012).

Fourier Transform Infrared Spectroscopy

FTIR spectroscopy was performed using NICOLET 6700 FT-IR machine equipped with OMNIC software. Absorption spectrum for each sample was obtained by applying 10 µl of sample between 2 zinc selenium windows secured in a holder. Every sample spectrum was background subtracted. Spectra were recorded at RT. All spectra were corrected for background spectrum of D_2O. Normalized spectra were plotted from 1500 cm^{-1} to 1700 cm^{-1} wavelength with major focus on amide I region ranging from 1600 cm^{-1} to 1700 cm^{-1} wavelength.

Mass Spectrometry

Trypsin Digestion of α-Syn Oligomers

The α-Syn monomer remained in SynO-DA and Syn-DHA preparation was removed by a microcentrifuge filter unit (molecular cutoff 30kDa) (Millipore). Ten micrograms of SynO-DA and Syn-DHA were added into a filter unit, respectively. Then 200 µl of 25 mM ammonium bicarbonate (pH 8.0) was added into each filter unit and centrifuged at 12,000 xg for 10 min. This step was repeated twice. The α-Syn oligomers remained in the filter was transferred into a 0.6-mL tube and 0.2 µg of trypsin was added into each sample. The sample was incubated at 37 ºC for 0.5, 1 and 5 h.

Parallel Reaction Monitoring (PRM) Analysis of *Rickettsia* Protein RC0497

For PRM analyses, the peptides were analyzed with Easy nLC1000 UHPLC -Q Exactive Orbitrap LC-MS system (Thermo Scientific, San Jose, CA). A 1-h linear gradient from 2% solvent A (0.1% formic acid in water) to 35% solvent B (0.1% formic acid in acetonitrile) was used for each LC-MS/MS run. The resolution of full scan was 70,000 (@m/z 200), the target AGC value was set to $3x10^6$, and maximum fill time was 200 ms for full scan; 17,500

(@m/z 200), a target AGC value of $2x10^5$, and maximum fill times of 100 ms for MS2 scan. PRM targeted eight tryptic peptides of α-Syn. The assessment of the detection of peptides was performed post-acquisition using Skyline version 3.6.0.9321 (MacLean, Tomazela et al. 2010, Egertson, MacLean et al. 2015). For each peptide evaluated, the signals of the 5-6 most intense fragment ions were extracted from each corresponding MS/MS spectrum. The MS/MS spectra of the fragment ions detected were submitted to spectral matching. The comparison of the relative intensities of these fragments with those defined in the reference composite MS/MS spectrum was performed based on the dot product (dotp) value.

Primary Cortical Neuron Culture

The C57BL/6 animals (Jackson Laboratory, 000664) were used for primary cortical neuron isolation. Animals were maintained in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. All required procedures were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for primary neuron isolation was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB).

Primary cortical neuronal cells from C57BL/6 mice during embryonic days 16-18 were isolated using Accutase solution (Sigma, A6964) and maintained as described elsewhere (Beaudoin, Lee et al. 2012). Neuronal cells were plated in a 24-well plate with poly-d-lysine-coated glass coverslips (Corning, Inc.) at a density of 2×10^5 cells/mL. Neurobasal medium (Gibco, 12348017) supplemented with 2% B-27, 0.5 mM GlutaMax (Gibco, 35050-061), 10,000 units/mL penicillin, 10,000 μg/mL streptomycin, and 25

μg/mL amphotericin B supplement was used to culture the cells. Half of the Media changes were performed every 3-5 days by replacing 50% culture media with fresh media. Cells were grown for 10-13 days in vitro (DIV) before experiments.

Cell Transfection and Treatment with α-Syn Oligomer Strains

EGFP/Puromycin-selective empty plasmid and EGFP/Puromycin-hSNCA (human wild type α-Syn; NM_00146054.1) expression plasmids were designed, generated and purified by VectorBuilder (Chicago, IL). Human neuroblastoma SH-SY5Y cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). After plating on coverslips, cells were transiently transfected with either EGFP/Puromycin-hSNCA or EGFP/Puromycin-selective empty plasmid DNA using Lipofectamine 2000 (Invitrogen). Briefly, an empirical concentration of plasmids (125 ng) were mixed with Lipofectamine 2000 (2 μl) for 30 min at RT followed by incubation with cells in FBS-deprived DMEM. After 6 h, culture medium was replenished with 5% FBS-supplemented DMEM for 16h. The next day, cells exposed to different α-Syn oligomeric strains (SynO-DA and SynO-DHA) at concentration of 0.125 and 0.25 μM. Cells were also treated with vehicle (empty vector) alone that were used as negative control. Three independent replicate experiments were performed for each experimental condition. Images were captured with a Keyence BZ-800 Microscope and analyzed using BZ-X Analyzer. A Nikon 100X oil immersion objective was used for image acquisition.

Cell Toxicity Assays

Cell toxicity and cell viability were determined in human neuroblastoma SH-SY5Y cells as well as SH-SY5Y cells overexpressing human wild type α-Syn, SH-SY5YWT-Syn. Both cell types were cultured and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco, 16000-044) and 1% penicillin/streptomycin (Gibco). Cytotoxicity was evaluated by measuring lactate dehydrogenase (LDH) release using Cytotoxicity Detection kit PLUS (Roche, 04744926001) and cell viability was measured by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, G5421) following manufacturers' instructions. In brief, cells were treated with five different concentrations of α-Syn oligomers: SynO-DA, SynO-DHA and SynO-UM, as well as fibrillar α-Syn ranging from 0.125 to 1.5 μM and incubated for 16 and 24 h followed by assaying with LDH. Cell viability assay was performed only at 24 h of incubation. For both assays, absorbance was measured at 490 nm with a Polar Star Omega plate reader (BMG Labtech). Each experimental condition was performed in triplicates in three different independent assays. For the MTS assay, the percentage of viable cells was calculated as $((OD_{\text{untreated control}} - OD_{\text{treated}})/ OD_{\text{untreated control}}) \times 100$. For LDH assay, the percentage of affected cells was calculated following the formula provided by the manufacturer.

Primary cortical neurons grown 96-well plate were treated with increasing concentrations of the three α-Syn oligomer preparations and α-Syn fibrils (0.05, 0.125, 0.25, 0.5, 1.0 and 1.5 μM) for 16 h. Additionally, we have also treated the neurons with α-Syn monomer for the same time period. The cytotoxicity was measured by evaluating LDH release.

Primary Neurons Treatment, Immunostaining and Confocal Microscopy

Primary cortical neurons grown on coverslip in a 24-well plate were exposed to 0.5 μM α-Syn oligomer strains for 6 h. For vehicle treated group, 1 X PBS was added to the neuronal cells and incubated for the same time period. After 6 h of incubation, cells were washed 3 times with 1X PBS and fixed with 4% formaldehyde solution for 15 min at RT. Cells were then washed 3 times with 1 X PBS followed by permeabilizing with 0.25 % Triton X-100 in PBS for 10 min at RT. Cells were blocked in 5% goat serum for 30 min at RT and incubated with primary antibodies, rabbit PSD95 (1:1000; Abcam, Ab18258) and mouse anti-β-III tubulin (1:1000; Abcam, ab78078) at 4°C overnight. Next day, cells were washed and incubated with Alexa fluor anti-rabbit 568 and Alexa fluor anti-mouse 488 (1:1000, Life Technologies) secondary antibodies for 1 h at RT. Following 3 washes, coverslips were mounted using ProLong Diamond antifade mounting media with DAPI (Invitrogen). Coverslips with all treatment conditions were imaged under Zeiss LSM 880 confocal microscope using 63x objective with 405 nm diode laser and argon laser 458/488/514nm. Z-stacks were built by capturing images from 17 stacks at 0.37-0.41 μm optimal thickness. Each treatment condition was performed in 3 independent experiment and were randomly imaged at five different regions of interest. All images were analyzed by ImageJ (NIH) software.

Dendritic Spines Analysis

To assess the effects of different α-Syn oligomeric polymorphs on the number of mature synapses, we followed our previously published method (Castillo-Carranza, Guerrero-Munoz et al. 2018). Briefly, primary cortical neuronal cells from embryos of C57BL/6 mice were exposed to vehicle (PBS), SynO-DA and SynO-DHA. Neuronal cells were then immunostained with PSD95 antibody (Abcam, Ab18258), a post-synaptic density marker

protein and βII-tubulin antibody (Abcam, ab78078), a neuronal marker protein. Five different areas of 20 μm dendritic shafts (without any branches) from each treatment were randomly chosen to count the PSD95 puncta. Images were taken from 5 different cells per treatment group using identical laser power, photomultiplier gain, and pinhole settings for each experiment. Images were analyzed by a researcher who was kept blinded to the experimental conditions. All treatment conditions were imaged under Zeiss LSM 880 confocal microscope using 63x objective with 405 nm diode laser and argon laser 458/488/514nm. Three independent experimental replicates were performed for each experimental setting. The intensity of mean PSD95 puncta was calculated using ImageJ software (NIH, Bethesda, Maryland, USA). The threshold value for each channel was set same across all experimental conditions. Intensity of PSD95 was determined by subtracting the background. One-way ANOVA followed by Tukey's post hoc was used to analyze the dendritic spine results.

Internalization of α-Syn Oligomer Strains

Primary cortical neurons from embryos of C57BL/6 mice were plated in 96-well plates at 4 X 10^4 cells/mL and exposed to Dynasore hydrate (6.5-26 μg/mL; Sigma, D7693) or Heparin (50-200 μg/mL; Sigma, H4784) for 30 min. Oligomeric α-Syn strains, SynO-DA and SynO-DHA were added to the cells at 1 μM concentrations and incubated for further 16 h. For each α-Syn oligomeric strain, two different inhibitors were used at three different concentrations (Rodriguez, Marano et al. 2018). Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using Cytotoxicity Detection kit PLUS (Roche, 04744926001).

Statistical Analysis

All statistical analyses were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). All values were calculated as mean and standard deviation (SD). Data are presented from at least 3 replicates and from 3 independent experiments. For cytotoxicity assay, average fluorescent intensity measurement and FRET positive cells quantification, two-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed. For bis-ANS and thioflavin T fluorescence assays, dendritic spine analysis, one-way ANOVA with Tukey's multiple comparisons test was performed. The number of experiments is mentioned in the figure legends.

RESULTS

Characterization of DA- and DHA-modified α-Syn Oligomers

We have generated oligomers of α-Syn protein by separately modifying the protein with DA and DHA and have thoroughly characterized them to evaluate their polymorphic nature. To generate polymorphic α-Syn oligomeric assemblies, we used purified human recombinant α-Syn protein as previously published (Sengupta, Guerrero-Munoz et al. 2015). Aggregates of α-Syn were prepared by modifying with DA at 1:20 molar ratio of protein to DA following published method by Lee et al, (Lee, Baek et al. 2011, Planchard, Exley et al. 2014). Upon oxidation, DA forms dopamine quinones (DAQs) which interact with α-Syn forming adducts that finally results in the formation of oligomeric structures (**Figure 2.1a**). The second condition used for α-Syn oligomerization was by modifying the protein with DHA at 1:50 molar ratio (protein: DHA). Studies have suggested that such modification is mostly based on covalent interaction as well (**Figure 2.1b**). Oligomers of α-Syn formed by modifying with DA and DHA are termed as SynO-DA and SynO-DHA,

respectively. For comparison purposes in biochemical analyses, we also prepared α-Syn oligomers (SynO-UM) without any modification, following our previously published method (Sengupta, Guerrero-Munoz et al. 2015). Fibrillar assemblies of human recombinant α-Syn (Syn fibrils) were used to compare all the three oligomeric preparations.

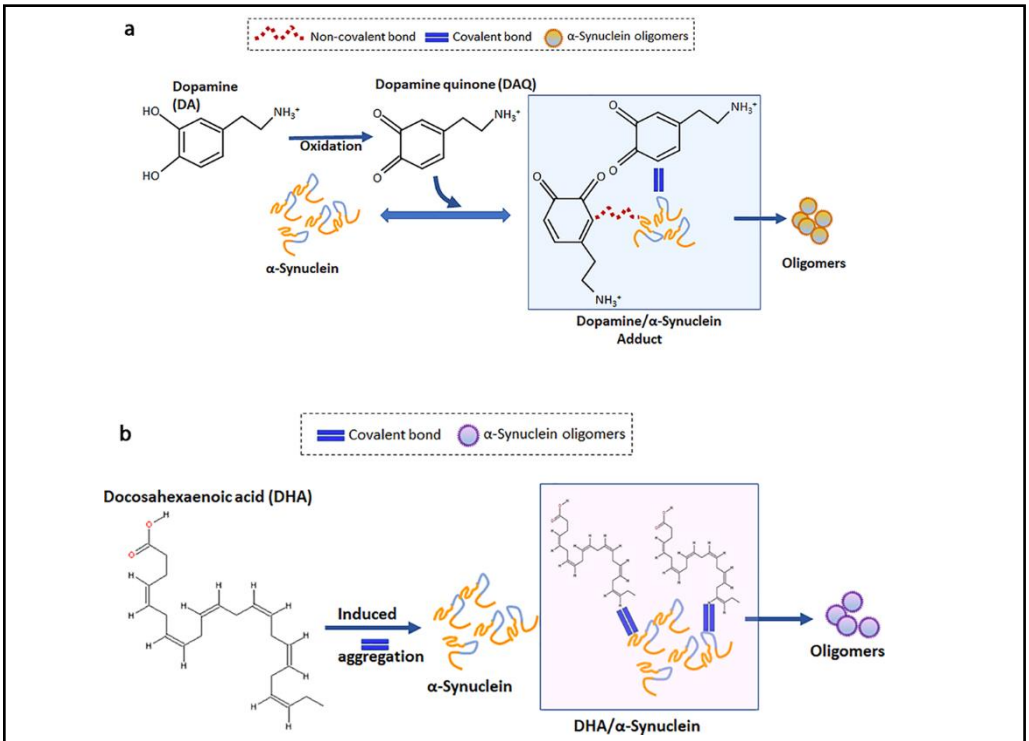

Figure 2. 1 Schematic representation of two recombinant α-Syn oligomeric polymorphs.
(**a**) Generation of α-Syn oligomers by Dopamine (DA) modification. Dopamine gets readily oxidized into its quinones which is then thought to interact with α-Syn by both covalently and non-covalently forming adducts. These adducts result in the formation of modified α-Syn oligomers, hence termed as SynO-DA. (**b**) Generation of α-Syn oligomers modified by docosahexaenoic acid (DHA), termed as SynO-DHA, primarily via covalent bonding.

SDS-PAGE gel electrophoresis of SynO-DA, SynO-DHA, SynO-UM and Syn fibrils followed by Western blot (WB) analyses with a generic α-Syn antibody, LB509 was performed. We used three different concentrations (0.6, 0.3 and 0.15 µg) of each

oligomeric and fibril preparation. WB analysis of SynO-DA showed high molecular weight (HMW) oligomers, mostly ranging from 50 kDa and above (**Figure 2.2a**). This was also evident from the size exclusion chromatography (SEC) of SynO-DA that exhibited a single dominant peak, indicating α-Syn oligomers (**Figure 2.2e**). DHA-modified α-Syn oligomers showed aggregates starting from dimer at ~28 kDa to higher than 250 kDa in WB analysis with LB509 antibody (**Figure 2.2b**). Apart from dimers, oligomers formed in this condition were visible as distinctive bands at ~50, ~70 and ~75 kDa that are indicative of different aggregate species. In the SEC chromatogram, SynO-DHA separated into 2 peaks of mostly HMW aggregates, followed by a peak of lower molecular weight (LMW) (**Figure 2.2f**). The unmodified α-Syn oligomers showed aggregates of different molecular weights in WB analysis with LB509 antibody as well in SEC (**Figure 2.2c, g**). The WB image of syn fibrils showed a strong band at the top which is usually noticeable in amyloid fibrils (**Figure 2.2d**). However, it also showed a few weak HMW bands of aggregates. Together, the results from the WB and SEC analyses of different preparations of α-Syn oligomers showed that they have different populations of aggregates. We studied the morphology of the oligomers modified by DA and DHA by atomic force microscopy (AFM) (**Figure 2.2h**). Both the conditions resulted in spherical α-Syn oligomers as shown by AFM images. The unmodified α-Syn oligomers showed similar spherical structures. Fibrillar α-Syn sample mostly showed protofibrils as well as long fibrils. The size distribution histograms showed that most of the SynO-DA oligomers had a height of 1.5-2 and ~ 3.75 nm, while the SynO-DHA oligomers had 1.5-2.5 nm (**Figure 2.2i**). The height of SynO-UM and α-Syn fibrils were mostly between 2.5-3.5 nm and 2-5.5 nm, respectively (**Figure 2.2i**). To assess the hydrophobicity and aggregation state of the two oligomeric preparations, we

performed fluorescence binding assays using bis-ANS and thioflavin T (ThT). It has been

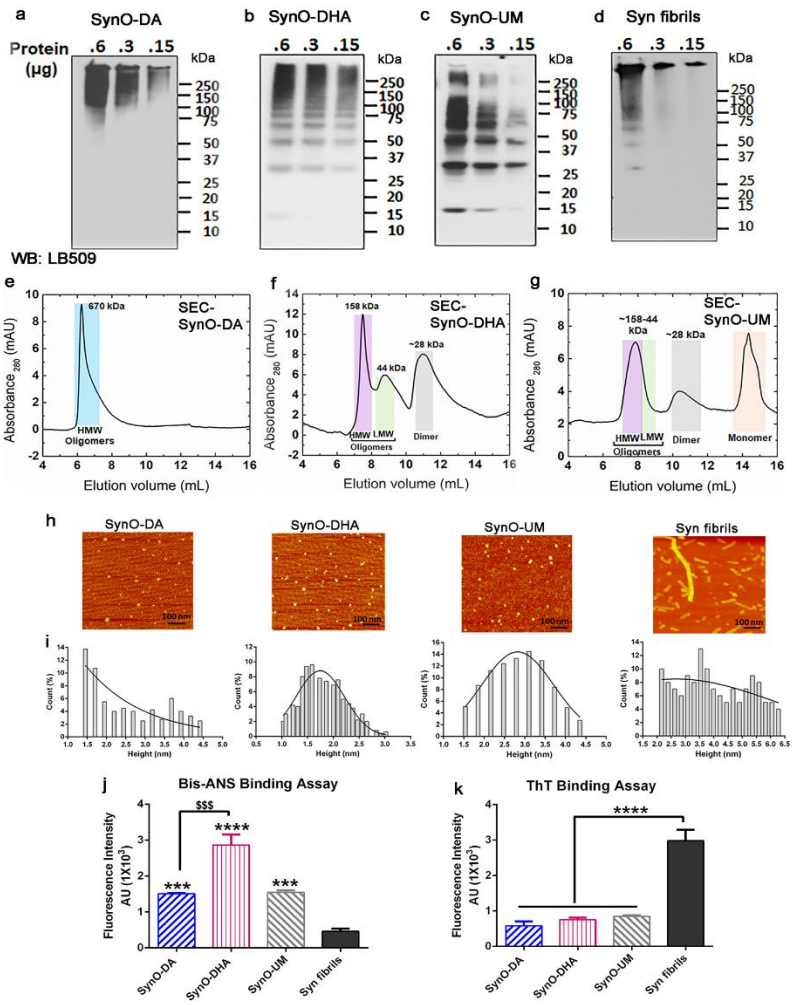

Figure 2. 2 Biochemical characterization of DA- and DHA-modified α-Syn oligomers. (**a, b**) Representative Western blot images of SynO-DA and SynO-DHA with different amounts of proteins immunolabeled with a generic α-Syn antibody, LB509. These two oligomers clearly show different orders of aggregates. (**c, d**) Western blot images of unmodified α-Syn oligomer (SynO-UM) and Syn fibrils probed with LB509 antibody. (**e-g**) Size exclusion chromatograms of the three oligomers showing different sizes of aggregates. HMW= high molecular weight, LMW= low molecular weight. (**h, i**) Representative AFM images of α-Syn aggregates with their height distribution. Scale bar 100 nm. (**j**) Fluorescence intensity of bis-ANS binding to α-Syn aggregates shows significantly strong binding to all the three α-Syn oligomers compared to Syn fibrils. (**k**) Fluorescence intensity of ThT binding to α-Syn aggregates. ThT binding to all three α-Syn oligomer preparations is significantly less compared to Syn fibrils. Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparisons test. $^{\$\$\$}$ $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$.

shown that bis-ANS fluorescent dye strongly binds with amyloid oligomers compared to

fibrils, while ThT binds strongly with amyloid fibrils rather than with oligomers (Sengupta, Guerrero-Munoz et al. 2015, Lo Cascio and Kayed 2018). In our study, both DA-modified and DHA-modified α-Syn oligomers showed comparable hydrophobicity upon binding with bis-ANS which was significantly higher than fibrils (**Figure 2.2j)**. However, SynO-DHA showed marginally increased affinity for bis-ANS than SynO-DA and SynO-UM. As expected, all three α-Syn oligomers showed low binding affinity for ThT compared to α-Syn fibrils (**Figure 2.2k**). Taken together, α-Syn oligomers prepared in presence of DA and DHA show differences in their biochemical properties.

DA- and DHA-modified α-Syn Oligomers are Structurally Distinct

To acquire insight into the structural properties of the two oligomeric α-Syn polymorphs, we determined their secondary structures by spectral analyses using fourier transform infra-red (FTIR) and circular dichroism (CD). The CD spectrum of DA-modified α-Syn oligomers exhibited mostly a random coil structure with a minimum around 195 nm (**Figure 2.3a**). The deconvoluted spectrum showed that in addition to random coil, this preparation also contained ~8.6% α-helix and ~26.12% β-sheet structures. On the other hand, α-Syn oligomers modified by DHA, showed α-helical structure as its major secondary constituent with two minima around 215 nm and 222 nm (**Figure 2.3a**). The deconvoluted spectrum showed ~56.82% α-helix and ~5.09% β-sheet structures in this oligomeric population. These observations are consistent with previous studies, where DA-modified oligomers mostly contained random coil and DHA modified oligomers contained α-helix as the main structural component. The CD spectrum of SynO-UM showed a distinct minimum around 195 nm indicating random coils with a maximum around 220 nm, indicative of β-sheet. On the other hand, α-Syn fibrils had two minima at approximately

206 nm and in the vicinity of 220 nm with a maximum around 196 nm with ~28.84% α-helix content and 13.66% β-sheet structures (**Figure 2.3b**). The CD spectra with a minimum around 218 nm and maximum at 196 nm were shown for α-Syn aggregates containing β-sheet structures. α-helix rich proteins were shown to have a minimum at 208 or 206 nm and a maximum around 193 nm. Our observation here is in accordance with a study, where both WT and mutant α-Syn proteins were shown to form α-helix rich oligomers and protofibrils as intermediary aggregates prior to β-sheet rich mature fibrils (Ghosh, Singh et al. 2015). The fibrillar α-Syn preparation used in this study mostly contained protofibrils, as shown by the AFM image (**Figure 2.2h, i**), which supports our observation in the CD analysis.The second derivatives of FTIR spectra for amide I regions of both DA-modified and DHA-modified oligomers showed a major peak. However, spectral region from 1600 cm^{-1} to 1700 cm^{-1} (insets) detailed the differences between the secondary structures of these oligomers (**Figure 2.3c, d**). The DA-modified oligomers showed a characteristic peak for random coil structure around 1648-1650 cm^{-1}, followed by another small peak at 1675-1685 cm^{-1}, that mostly indicates β-turn (Yang, Yang et al. 2015). Moreover, SynO-DA showed a small peak at 1530 cm^{-1} which falls in the amide II region and such spectrum has been associated with β-sheet structures (Zandomeneghi, Krebs et al. 2004) (**Figure 2.3c**). DHA-modified oligomers displayed a peak at 1652 cm^{-1}, resulting mostly from α-helix followed by a deep shoulder at 1695 cm^{-1}, representing β-sheet structure (**Figure 2.3d**). Additionally, we noticed a peak at around 1614 cm^{-1}, indicating a cross-β structure as detailed in the inset (marked by a black line) of expanded spectra from 1600 to 1700 cm^{-1} for amide I region, in accordance with previous study (De Franceschi, Fecchio et al. 2017). SynO-UM mostly showed random coils with an

absorption spectrum around 1649 cm^{-1} (**Figure 2.3e**), whereas, α-Syn fibrils showed an enlarged peak in the vicinity of 1630-1656 cm^{-1} (**Figure 2.3f)**. FTIR spectra around 1631-1635 cm^{-1} and around 1653-1656 have been assigned to β-sheet and α-helical structures of

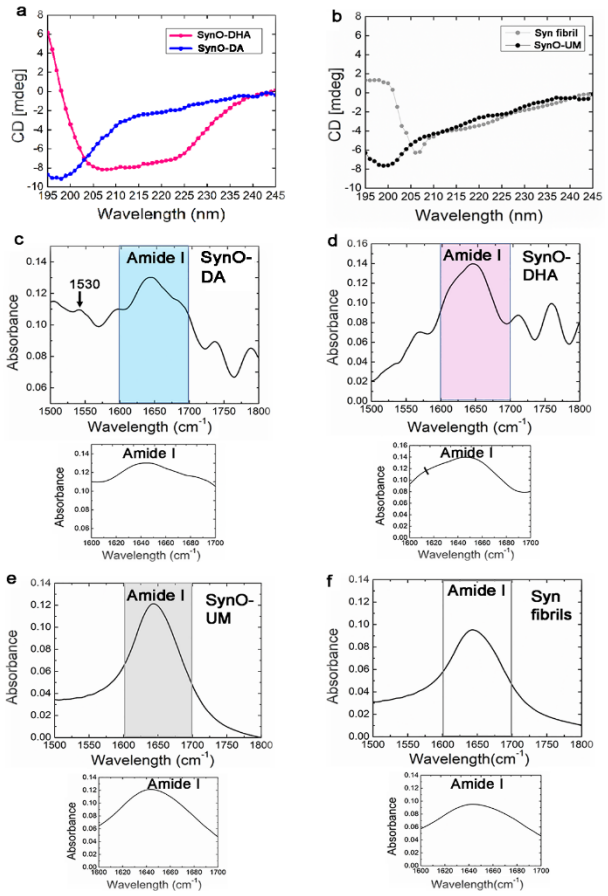

Figure 2. 3 Biophysical Characterization of DA- and DHA-modified α-Syn oligomeric polymorphs.

(**a**) CD spectra of SynO-DA and SynO-DHA. SynO-DA shows a minimum around 195 nm indicating mostly random coil, whereas, SynO-DHA showed two minima at 215 and 222 nm, suggesting α-helical structure. (**b**) CD spectra of SynO-UM showed random coil and β-sheet, whereas, Syn fibrils showed α-helical and β-sheet structures. (**c**) FTIR spectrum of SynO-DA with the inset showing 1600 to 1700 cm^{-1} corresponding to the amide I region. This oligomer preparation mostly contains random coil with a peak around 1648-1650 cm^{1}. A small absorption peak at 1530 cm^{-1} in amide II region corresponds to β-sheet structure (marked by black arrow). (**d**) FTIR spectrum of SynO-DHA shows α-helical structure with an absorption at 1652 cm^{-1}. It also indicates a cross-β-structure (1614 cm^{-1}), marked by a black line in the inset. (**e-f**) FTIR absorption spectrum of SynO-UM mostly showed random coil, while an enlarged peak around 1630-1656 cm-1 was observed for Syn fibrils, indicative of β-sheet and α-helical structures.

α-Syn aggregates, respectively. Therefore, results from CD and FTIR spectroscopic analyses indicate that SynO-DA and SynO-DHA are two structurally distinct α-Syn oligomeric polymorphs.

Oligomeric α-Syn Polymorphs Exhibit Differential Toxicity and Dendritic Spine Pathology

Exogenously added different types of α-Syn oligomers were shown to cause cellular toxicity either by seeding endogenous protein or by acting on cellular membranes, thus elevating intracellular calcium influx (Danzer, Haasen et al. 2007). We anticipated that our different oligomer preparations might not possess similar potency to cause cellular toxicity. Therefore, next we sought to assess the dose and time-dependent toxic effects of the two α-Syn oligomers by exogenously adding them to human neuroblastoma cells, SH-SY5Y and the same cell line overexpressing human wild type (WT) α-Syn protein (SH-SY5Y$^{WT\text{-}Syn}$). We used five different concentrations of SynO-DA and SynO-DHA as well as SynO-UM ranging from 0.125 to 1.5 µM and incubated for 16h and 24h followed by measuring lactate dehydrogenase (LDH) release (**Figure 2.4**). To compare the toxicity of the three α-Syn oligomers, α-Syn fibrils were used at the same concentrations and incubated for the same time points. Release of lactate dehydrogenase (LDH) by the α-Syn aggregate treated cells was measured to estimate toxicity. We observed a dose- and time-dependent change in the levels of LDH release in all the oligomers treated groups. In SH-SY5Y cells, SynO-DHA and SynO-UM showed dose-dependent toxicity which was significantly increased compared to the α-Syn fibrils and was maximum at 24 h of incubation. Although SynO-DA showed a linear dose-dependent increase in LDH release, it did not cause comparable toxicity in these cells at 16 h of incubation. At 24 h of

incubation, all three oligomers showed toxicity compared to α-Syn fibrils (**Figure 2.4a, b**). In SH-SY5Y^{WT-Syn} cells, both SynO-DA and SynO-DHA showed significantly increased

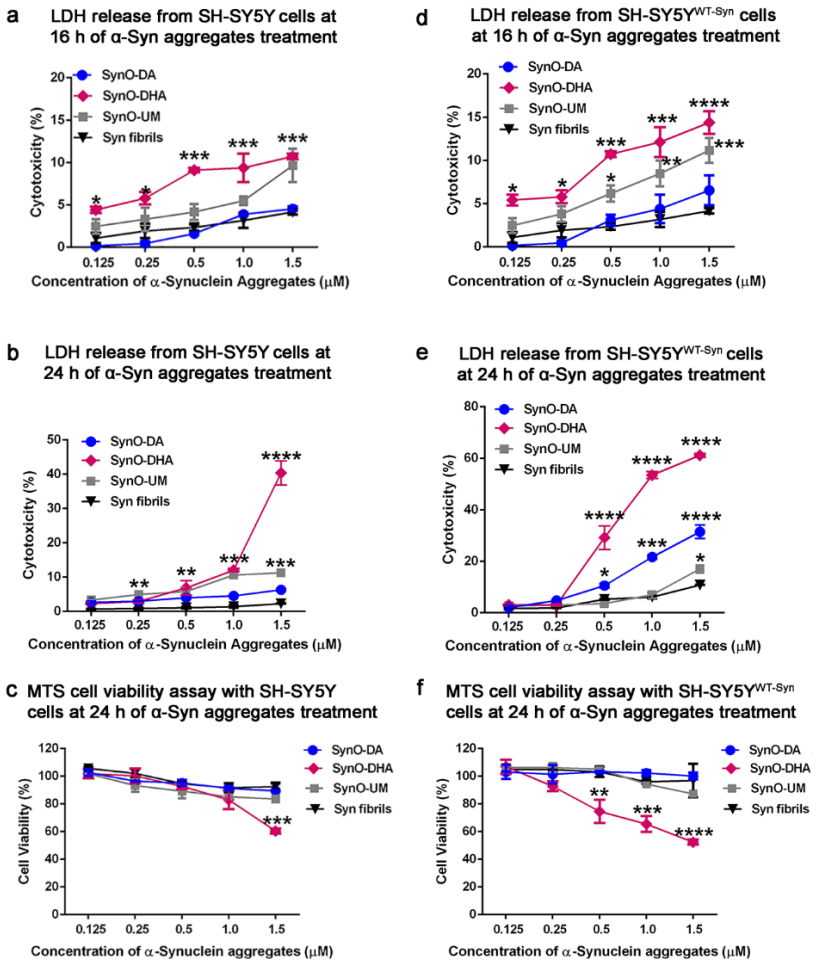

Figure 2. 4 Dose- and time-dependent cytotoxicity induced by α-Syn oligomeric polymorphs.
SH-SY5Y cells and SH-SY5Y cells overexpressing human wild type α-Syn protein, SH-SY5Y^{WT-Syn} were exposed to 0.125 to 1.5 μM of different α-Syn oligomers preparations for the indicated times. (**a, b**) The effect of different concentrations of α-Syn aggregates in SH-SY5Y cells as measured by LDH release at 16 and 24 h of incubation. (**c**) Cell viability of SH-SY5Y cells exposed to different concentrations of α-Syn aggregates for 24 h measured by MTS assay. (**d, e**) The effect of α-Syn aggregates in SH-SY5Y^{WT-Syn} incubated for 16 and 24 h as measured by LDH release. (**f**) Cell viability of SH-SY5Y^{WT-Syn} cells exposed to different concentrations of α-Syn aggregates for 24 h, measured by MTS assay. Data are represented as mean ± SD from three independent experiments. Statistical significance was calculated using two-way ANOVA with Bonferroni post hoc analysis. * $p<0.05$, ** $p<0.01$, *** $p< 0.001$, ****

dose-dependent toxicity. Interestingly, SynO-DA was more toxic than SynO-UM (**Figure**

2.4e). Additionally, we estimated the viable cells following oligomers treatment for 24 h by MTS assay. We observed reduced viability of cells exposed to SynO-DHA, consistent with cytotoxicity We observed reduced viability of cells exposed to SynO-DHA, consistent with cytotoxicity assay (**Figure 2.4c, f**). It is noteworthy to mention that, we observed an increased cytotoxicity and a decreased cell viability in the SH-SY5Y$^{WT\text{-}Syn}$ cells (**Figure 2.4e, f**) compared to the SH-SY5Y cells (**Figure 2.4b, c**) at 24 h of oligomers treatment. This augmentation in the oligomer-mediated cytotoxicity in SH-SY5Y$^{WT\text{-}Syn}$ cells might be driven by the overexpression of the α-Syn protein. The cytotoxic effects of the two α-Syn oligomers were also examined by exogenously adding them to the primary cortical neurons isolated from wild type C57BL/6 mouse embryos. Primary neurons were exposed to SynO-DA, SynO-DHA, SynO-UM, α-Syn fibrils as well as α-Syn monomer at an increasing concentration (0.05, 0.125, 0.25, 0.5, 1.0 and 1.5 µM) for 16 h (**Figure 2.5a, b**). A dose-dependent increase in the levels of LDH release was noticed with increasing concentration of SynO-DA, SynO-DHA and SynO-UM compared to the α-Syn fibrils (**Figure 2.5a**) and α-Syn monomer preparations (**Figure 2.5b**).

To further assess the functional roles of the two α-Syn oligomeric polymorphs, primary cortical neurons from wild type C57BL/6 mouse embryos were exposed to vehicle (PBS), and the two α-Syn oligomeric polymorphs for 6 h. Cells from all groups were immunostained with antibody for postsynaptic density protein 95 (PSD 95) and βIII-tubulin antibody for neurons, followed by imaging with confocal microscopy (**Figure 2.5c-e**). Both the oligomeric polymorphs reduced the number of dendritic spines that were visualized as puncta of PSD-95 positive structures. However, SynO-DHA caused spine

reduction that was slightly higher than SynO-DA (**Figure 2.5f**). Taken together these data suggest that DA- and DHA-modified α-Syn oligomers have distinct cellular consequences.

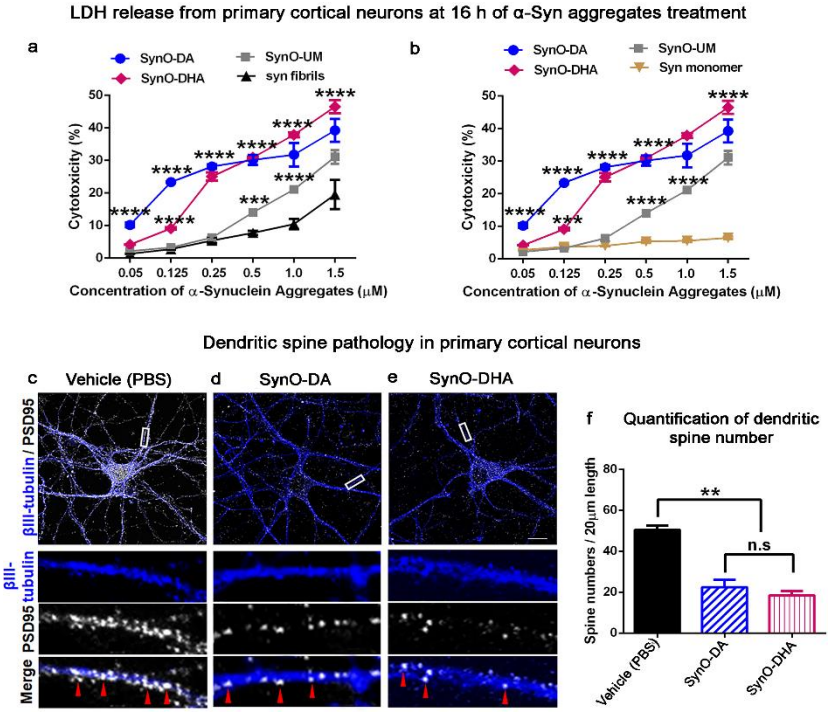

Figure 2. 5 Dose-dependent cytotoxicity and dendritic spine pathology in primary cortical neurons exposed to two α-Syn oligomer polymorphs.
(**a-b**) Cytotoxicity in primary cortical neurons from C57BL/6 embryos exposed to 0.125 to 1.5 μM of different α-Syn oligomers preparations for 24 h was determined by measuring LDH release (in percentage). Neurons exposed to SynO-DA and SynO-DHA showed significant toxicity compared to α-Syn fibrils (**a**) and α-Syn monomer (**b**). (**c-e**) Representative confocal microscopic images of primary cortical neurons from C57BL/6 embryos treated with SynO-DA and SynO-DHA for 6 h and immunolabeled with marker for postsynaptic density protein, PSD95 (appearing as white puncta) and β-III tubulin as neuronal marker (blue). The spines are marked by red arrowheads in the merged images. (**f**) Quantification of postsynaptic density protein 95 puncta calculated per 20 μm length of dendritic shaft. Primary neurons treated with the two α-Syn oligomer preparations show significantly decreased number of dendritic spines compared to the vehicle treated ones. The quantification is represented as mean ± SD from five randomly chosen areas of dendritic shafts from five different cells per treatment group in three independent experiments. One-way ANOVA with Tukey's multiple comparisons test. The significance is shown for SynO-DA and SynO-DHA with SynO-UM, which was ** $p < 0.01$ in both the cases. Scale bar 10 μm.

DA- and DHA-modified α-Syn Oligomeric Polymorphs Reveal Distinct Sensitivity to Proteinase K

In the previous sections, we have established that the two oligomeric polymorphs are different in their distribution of aggregate size, hydrophobicity and biological properties. Next, to evaluate the qualitative conformational differences between the two oligomeric polymorphs as well as their stability as oligomers, we measured their sensitivity for Proteinase K (PK) enzyme digestion. PK digestion had long been used in classifying strains of prion fibrils (Supattapone, Muramoto et al. 2001, Colby, Wain et al. 2010). Nevertheless, this method has been extended and widely used for identifying other amyloid strains, such as amyloid-β, α-Syn and tau fibrils (Guo, Covell et al. 2013). We treated SynO-DA, SynO-DHA and SynO-UM with increasing concentrations of PK enzyme (0-2 μg/mL). All the digested samples were then run in SDS-PAGE gel followed by silver staining to visualize the fragments. The pattern of fragments generated by PK digestion provides information on the stability of the oligomers, as well as its core. We observed α-Syn oligomers modified by DA were the most resistant to PK, thus indicating a stable core of these oligomers (**Figure 2.6a**).

By contrast, DHA induced α-Syn oligomers, SynO-DHA were sensitive to PK showing a fragmentation pattern which was different from the unmodified α-Syn oligomers, SynO-UM. To show the pattern of HMW aggregates following PK digestion which appeared in silver staining, we simultaneously performed WB analysis with the same set of PK-digested samples by immunolabeling with LB509 antibody (**Figure 2.6b**). The HMW bands that were more resistant to PK in the three oligomer samples, were detected based on their epitope availability for LB509 antibody. These HMW aggregates were

quantified and compared with the undigested counterparts for all the three samples (0 PK) (**Figure 2.6c**). The presence of PK-resistant HMW aggregates in WB analysis of SynO-DA was consistent with its silver staining result. Nonetheless, these HMW aggregates

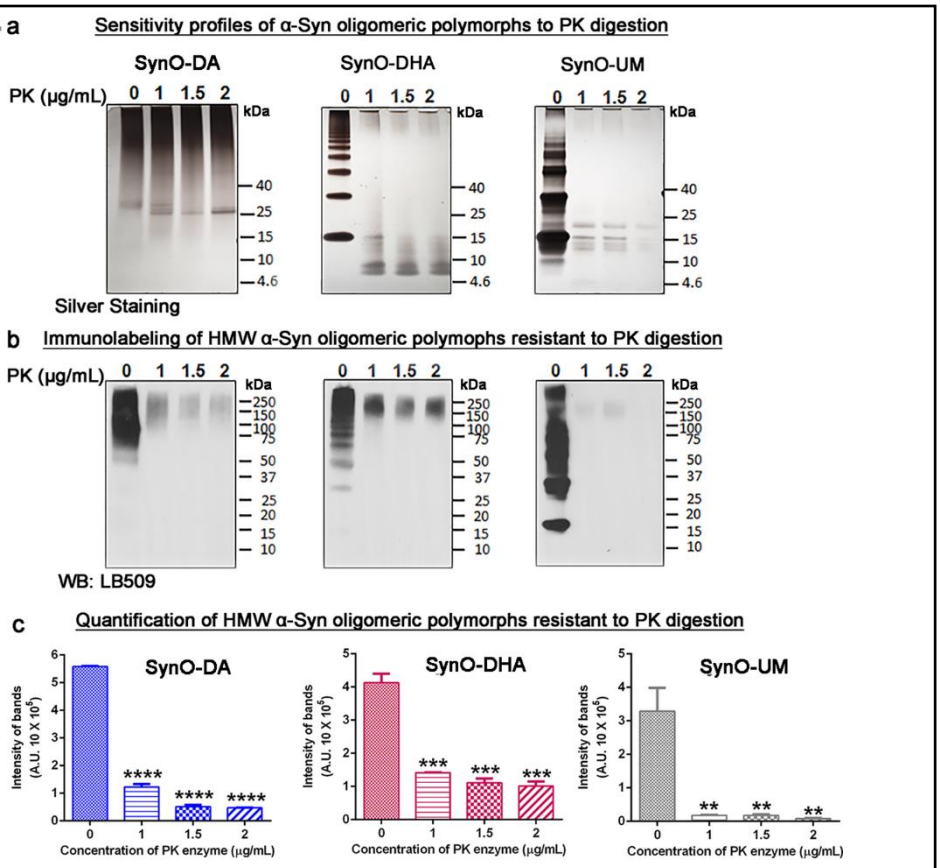

Figure 2. 6 Proteolytic digestion profiles of α-Syn oligomeric polymorphs. (**a**) Silver staining images of α-Syn oligomers, SynO-DA, SynO-DHA and SynO-UM digested with 1, 1.5 and 2 μg/mL Proteinase K (PK) enzyme. (**b**) Representative WB images of the same set of samples as in (**a**), immunolabeled with LB509 antibody, showing the PK-resistant high molecular weight (HMW) aggregates. (**c**) Densitometric quantification of the undigested bands (HMW aggregates) of b. Undigested bands from each sample are compared with the band from untreated sample (0 PK). Bars represent mean ± SD, calculated from three independent experiments. Statistical significance is calculated using one-way ANOVA with Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

showed decreased signal with increased concentration of PK enzyme. Similarly, SynO-DHA showed partially undigested HMW aggregates that decreased with increased concentration of PK enzyme. The signal for HMW aggregates in SynO-UM was less strong

in silver staining and in WB analyses. These data together indicate that SynO-DA and SynO-DHA are two distinct strains of α-Syn oligomers.

Furthermore, to support our observation from PK digestion, we performed mass spectrometry analysis of trypsin digestion of SynO-DA and SynO-DHA. The two α-Syn oligomer strains were digested with trypsin for 0.5, 1 and 5 h under native condition. The amino acid sequence of human α-Syn protein is shown in **Figure 2.7a**. The C-terminal sides of lysine and arginine amino acid residues are susceptible to trypsin cleavage. We analyzed eight tryptic peptides **(Figure 2.7b**) from SynO-DA and SynO-DHA with parallel reaction monitoring (PRM)-mass spectrometry (MS). The sequence coverage of the MS

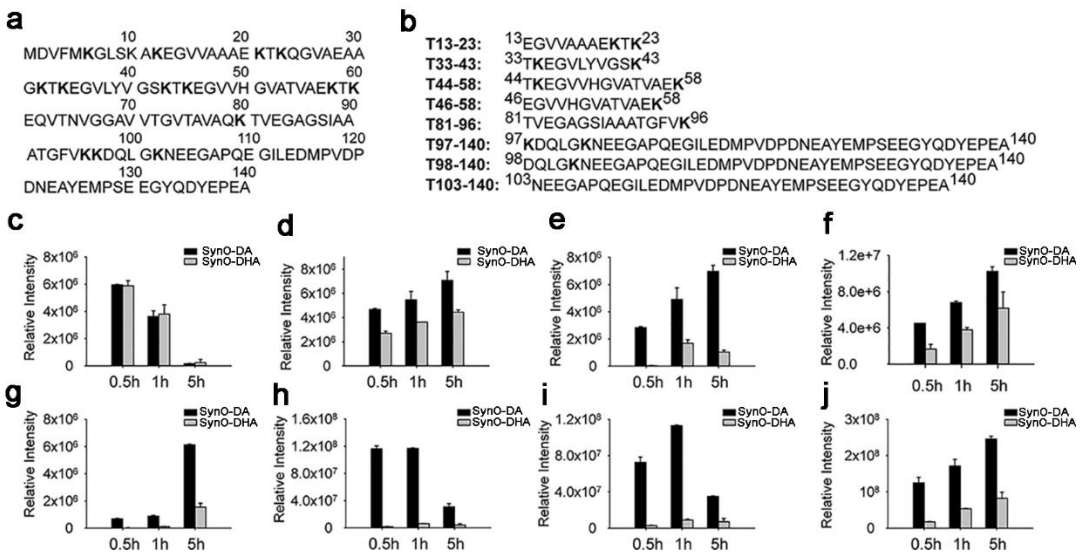

Figure 2. 7 Mass spectrometry analysis of trypsin digestion two α-Syn oligomeric polymorphs.
SynO-DA and SynO-DHA were digested with trypsin for 0.5, 1 and 5 h under native condition. The resulting tryptic peptides were analyzed by mass spectrometry. The intensity of each peptide was normalized with the proteotypic peptide of α-Syn, EGVLYBGSK. (**a**) The amino acid sequence of α-Syn. (**b**) Tryptic peptides of SynO-DA and SynO-DHA that were analyzed by LC-MS. The relative intensities of tryptic peptides T13-23 (**c**), T33-43 (**d**), T44-58 (**e**), T46-58 (**f**), T81-96 (**g**), T97-140 (**h**), T98-140 (**i**), and T103-140 (**j**).

analysis is 74% including N-terminal and C-terminal peptides of α-Syn, and the peptides in the middle of the sequence of α-Syn. The MS intensity of each peptide originated from

SynO- DA and SynO-DHA is shown in the **Figure 2.7c-j**. The abundance of the tryptic peptides of SynO-DA and SynO-DHA varies depending on the location of the peptide in the sequence of α-Syn and the types of inducer of α-Syn polymerization. Peptide T13-23 (EGVVAAAEKTK) is an N-terminal peptide. The MS intensities of this peptide originated from SynO-DA and SynO-DHA were almost same (**Figure 2.7c**), suggesting that the trypsin-accessibility to the lysine residues on N-terminal of SynO-DA and SynO-DHA are very similar. On the contrary, the trypsin-accessibility to the lysine residues on C-terminal of SynO-DA and SynO-DHA are significantly different. For example, after 0.5 h of digestion with trypsin, SynO-DA produced about 64 times more C-terminal peptide T97-140 than SynO-DHA (**Figure 2.7h**), implying the conformation of SynO-DA may hinder the accessibility of Lys92 to trypsin. We observed the similar phenomenon for C-terminal peptide T98-140. The lysine residues in the middle of α-Syn also displayed some differences in trypsin-accessibility (**Figure 2.7d-g**). These results suggest that the conformational differences between SynO-DA and SynO-DHA differentially effect the proteolysis of the oligomers.

α-Syn Oligomeric Strains Show Different Seeding Potencies of Cytosolic α-Syn protein

One of the major phenomena in amyloid strains is that the strains act as seeds in the recipient cells, thus recruiting endogenous protein into the aggregation, thus, augmenting the degeneration of cells. Therefore, to investigate if the two α-Syn oligomeric strains have seeding potency or not, we used SH-SY5Y cells transiently transfected to express human wild type α-Syn protein linked to enhanced green fluorescence protein (EGFP-hSyn), SH-SY5Y$^{EGFP\text{-}hSyn}$. Two different concentrations of SynO-DA and SynO-DHA (0.125 and 0.25

μM) were exogenously added as seeds to SH-SY5Y$^{EGFP\text{-}hSyn}$ cells and incubated for 16 h.

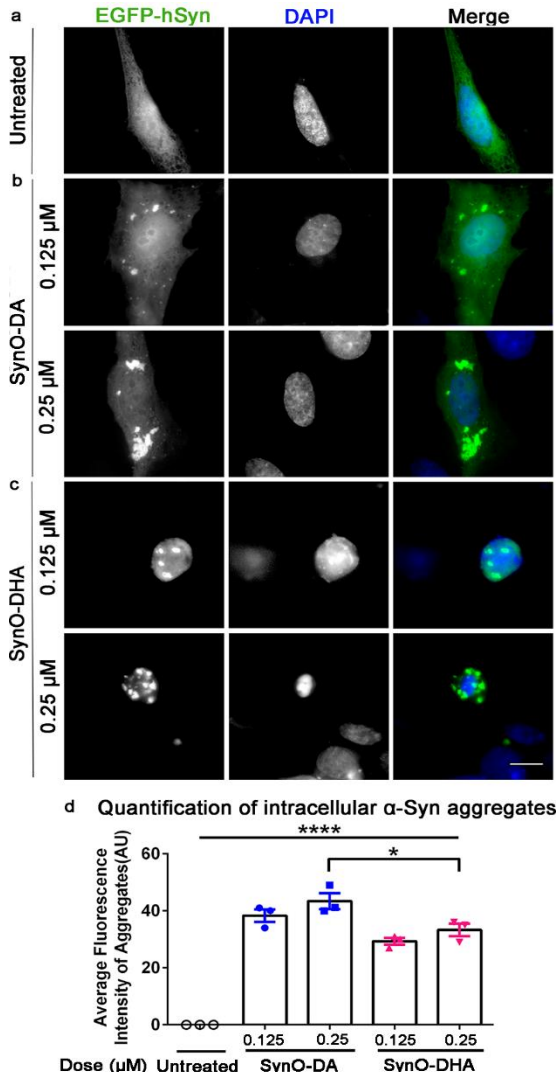

Figure 2. 8 Seeding potency of α-Syn oligomeric strains.
(**a-c**) Representative epifluorescence microscopic images of SH-SY5Y cells transiently expressing EGFP-hSyn that were exposed to SynO-DA and SynO-DHA at 0.125 and 0.25 μM concentrations for 16 h. EGFP-hSyn (green) and DAPI (blue; nuclei) are shown in grey. The merged images on right panels show cytosolic α-Syn aggregates formed by seeding with the different concentrations of α-Syn oligomeric strains: SynO-DA (**b**) and SynO-DHA (**c**). Cells expressing EGFP-hSyn but not exposed to exogenous α-Syn oligomers, do not show formation of aggregates. (**d**) Quantification of average fluorescence intensity of α-Syn aggregates calculated from fifteen different regions of interest (ROIs) in three different fields from five independent experiments. * $p < 0.05$, **** $p < 0.0001$. Two-way ANOVA with Bonferroni post hoc analysis. Scale bar 10 μm.

The dose of the oligomers and the incubation time point were chosen based on the toxicity

results shown in **Figure 2.4**. Strikingly, both SynO-DA and SynO-DHA were able to recruit cytosolic EGFP-hSyn protein into aggregates observed as bright green deposits (**Figure 2.8b, c**). No aggregation was seen in untreated cells (**Figure 2.8a**). Aggregates were observed as bright green deposits at both the two concentrations used (**Figure 2.8d**).

Dynamin and HSPG Antagonists Inhibit Internalization of α-Syn Oligomeric Strains and Reduce Oligomer-induced Cytotoxicity in Primary Neurons

We have demonstrated that the two α-Syn oligomeric strains act as seeds for cytosolic α-Syn protein aggregation. Next, we sought to investigate if the two oligomeric strains can be internalized into the cells via same or different mechanisms. We exposed primary cortical neurons to pharmacological inhibitors for dynamin-dependent (Dynasore) and Heparan sulfate proteoglycans (HSPGs)-mediated (Heparin) endocytic pathways. Cortical neurons were incubated for 30 mins in presence of three concentrations of each inhibitor as well as in the absence of any inhibitor. After the incubation period, cells were exposed to 1 μM α-Syn oligomeric strains, SynO-DA and SynO-DHA and incubated further for a total of 16 h. Toxicity induced by the α-Syn oligomeric strains in presence and absence of inhibitors, was assessed by detecting LDH release from the cell culture media (**Figure 2.9**). Both Dynasore and Heparin significantly blocked the internalization of the oligomeric strains, thereby, rescuing the cells from oligomer-induced toxicity. We observed less toxicity from SynO-DA with increasing concentration of these two inhibitors, indicating that DA-modified oligomers were internalized via dynamin- and HSPGs-mediated endocytosis (**Figure 2.9a)**. Dynamin-dependent internalization was more favored by these oligomers than HSPGs-mediated endocytosis, as observed from

reduced toxicity of SynO-DA in presence of Dynasore compared to Heparin inhibitor. Similarly, toxicity from SynO-DHA was significantly rescued in presence of both Dynasore and Heparin inhibitors. However, the Heparin appeared to block the

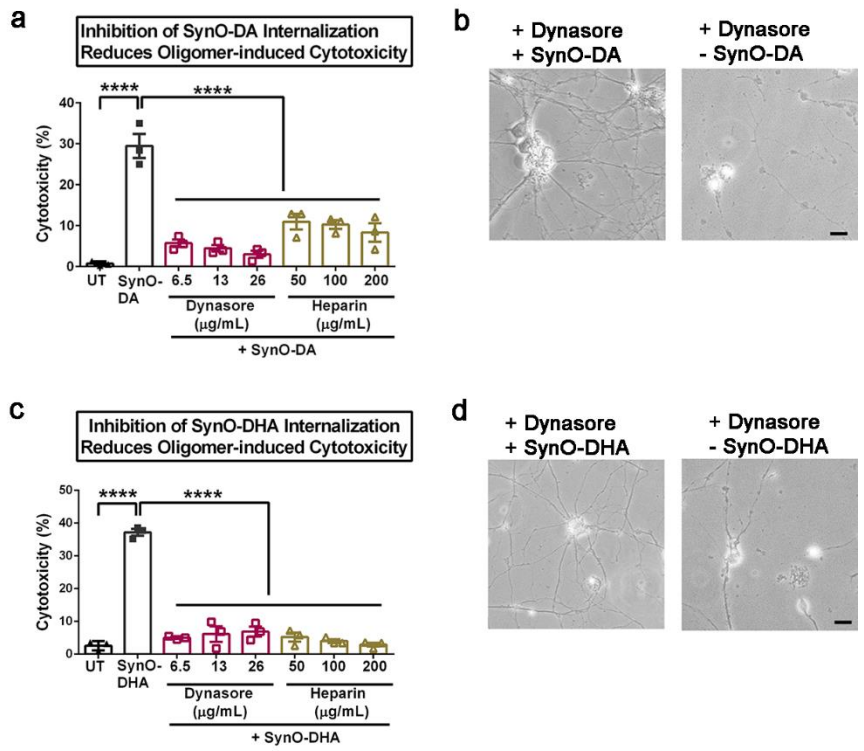

Figure 2. 9 HSPG and dynamin antagonists reduce α-Syn oligomeric strains internalization and cytotoxicity in neurons.
Primary cortical neurons were pre-treated with three different concentrations of the two inhibitors: Dynasore (6.5-26 µg/mL) and Heparin (50-200 µg/mL) for 30 min. α-Syn oligomeric strains, SynO-DA and SynO-DHA were exogenously added to the cells at 1.5 µM concentrations and further incubated for a total of 16 h. (**a, c**) Cytotoxicity induced by SynO-DA (**a**) and SynO-DHA (**c**) in absence and presence of the two inhibitors was assessed by measuring LDH release. Internalization of oligomers was blocked in presence of both the inhibitors, thus rescuing oligomers induced toxicity. (**b, d**) Representative live cell images of primary cortical neurons exposed to SynO-DA (**b**) and SynO-DHA (**d**) in presence and absence of the Dynasore inhibitor. Oligomer-induced toxicity was rescued when cells were treated in presence of Dynasore inhibitor. The quantification is represented as mean and SEM from three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test, **** $p<0.0001$. Scale bar 10 µm.

internalization of SynO-DHA slightly higher than Dynasore, suggesting that these oligomers favored HSPGs-mediated endocytosis than dynamin-dependent pathway

(**Figure 2.9c)**. Representative epifluorescence images of primary neurons treated with SynO-DA and SynO-DHA in absence and presence of Dynamin inhibitor (**Figure 2.9b, d**). We observed that the morphological alterations in primary neurons caused by the toxic effect of the oligomers were rescued when the cells were treated with oligomers but in presence of Dynamin inhibitor. These results suggest that, as the internalization of the α-Syn oligomeric strains are inhibited by the pharmacological inhibitors from being internalized into the cells, oligomer-associated cytotoxicity is prevented.

DISCUSSION

To date, several laboratories have demonstrated the polymorphic nature of amyloid fibrils, such as amyloid β and α-Syn fibrils as well as tau fibrils. However, polymorphism of α-Syn and tau in their toxic oligomeric conformation is still under investigation. In this study, we report the formation of two disease relevant α-Syn oligomeric strains modified by biological inducers such as, DA and DHA. The occurrence of oligomeric α-Syn and tau co-aggregates in PD and DLB brain tissues (Sengupta, Guerrero-Munoz et al. 2015), suggest the cross-seeding phenomenon. Therefore, here we have also demonstrated the effects of distinct α-Syn oligomeric strains on aggregation of tau protein. To the best of our knowledge, this is the first study demonstrating such distinct biologically relevant α-Syn oligomeric strains with differential consequences on tau aggregation by cross-seeding, thus resulting in discrete aggregated tau strains.

The reasons behind selective vulnerability of dopaminergic neurons being affected first in PD pathology, is not clearly known. However, one of the proposed mechanisms points to the possible modification of α-Syn by DA (Galvin 2006). A well-known

characteristic of α-Syn protein is its ability to interact with lipid membranes. It has been shown that α-Syn can form oligomers by interacting with DHA, an abundantly expressed PUFA in the brain (Sharon, Bar-Joseph et al. 2003). Here, we have demonstrated that the effects of DA and DHA modification on α-Syn oligomerization can lead to considerable polymorphism of α-Syn oligomers with differential biological effects. Biochemical analyses of these two preparations of α-Syn oligomers revealed differences in their aggregate size. The toxicity of oligomers is shown to correlate with increased surface hydrophobicity as measured by their binding to fluorescent probe bis-ANS as well as small size (Mannini, Mulvihill et al. 2014). A previous study has shown that treating SH-SY5Y cells expressing human α-Syn protein with polyunsaturated fatty acids can induce SDS-stable oligomers which cause cytotoxicity (Assayag, Yakunin et al. 2007). In our study, DHA modified α-Syn oligomers showed significant dose- and time-dependent increase in cytotoxicity than DA modified oligomers. A possible explanation could be that the surface hydrophobicity of DHA modified oligomers is higher than DA modified oligomers observed from bis-ANS fluorescence binding assay, despite their similar spherical morphology, as observed using AFM. From spectroscopic analyses, we observed that the two α-Syn oligomeric polymorphs have different secondary structures: DA modified oligomers mostly contain random coil, while DHA modified oligomers have α-helix as the main component. This observation is consistent with previous findings (Lee, Baek et al. 2011, Planchard, Exley et al. 2014, van Diggelen, Hrle et al. 2019). Moreover, both the oligomer preparations showed significant reduction of dendritic spines in mouse primary cortical neurons. This observation supports the toxic effects of the oligomers since

dendritic spine pathology as one of the most commonly occurring events in neurodegenerative diseases (Dorostkar, Zou et al. 2015, Herms and Dorostkar 2016).

Digestion of α-Syn oligomers with PK enzyme reveals differences in the sensitivity of DA and DHA modified preparations to proteolysis. DA modified oligomers show resistance to proteolysis, while, DHA modified oligomers are sensitive showing cleaved fragments, suggesting these two oligomeric polymorphs can be considered as strains. Additionally, tryptic digestion of the two oligomers followed by mass spectrometric analysis show different cleavage patterns among the oligomer preparations, further suggesting differences in their stability. Interestingly, both DA and DHA modified oligomers can seed cytosolic α-Syn protein into aggregates at different levels. However, further studies will be required to clearly understand the seeding effects of the two strains. Our observation of cytosolic protein seeding is in agreement with a previous study where DA modified α-Syn oligomers seeded cytoplasmic α-Syn in a reporter neuroblastoma N2A cell line (Pieri, Madiona et al. 2016). There is no study showing such seeding effect of DHA modified oligomers. It has been shown that extracellular α-Syn oligomers impair the lysosomal degradation pathway, leading to its intracellular accumulation (Hoffmann, Minakaki et al. 2019). Here we did not study the clearance mechanism of the two α-Syn oligomeric strains. Therefore, further studies in this aspect may provide more insight into the understanding of DA- and DHA-mediated α-Syn toxicity. Taken together, our biochemical and biophysical analyses suggest that DA- and DHA-modified α-Syn oligomers are two distinct strains with different conformation, stability and biological functionalities.

Recent studies have shown that cell-to-cell spreading of pathogenic protein aggregates is necessary for propagation of diseases (Lee, Desplats et al. 2010, Reyes, Sackmann et al. 2019). Heparan sulfate proteoglycans (HSPGs), a family of proteins containing one or more sulfated glycosaminoglycan (GAG) heparan sulfate (HS) have been shown to play an important role in cellular uptake of aggregated proteins, such as fibrils of amyloid-β (Kanekiyo, Zhang et al. 2011) and tau (Holmes, DeVos et al. 2013). Both fibrillary α-Syn and exosome-associated oligomeric α-Syn were shown to be internalized in cells via HSPGs (Holmes, Furman et al. 2014, Delenclos, Trendafilova et al. 2017). A recent study suggests that α-Syn oligomers can also be internalized in human neuroglioma cell line H4 via clathrin-mediated endocytic pathway (Hoffmann, Minakaki et al. 2019). Primary cortical neurons were shown to uptake oligomeric forms of α-Syn via dynamin-dependent clathrin-mediated endocytic pathway (Lee, Suk et al. 2008). Defects in clathrin-mediated endocytic pathway is considered as a susceptibility factor in PD and parkinsonism (Medeiros, Bubacco et al. 2018). In our study, since both the α-Syn oligomeric strains were able to successfully seed cytosolic α-Syn protein aggregation, we were interested in examining the internalization mechanisms favored by these oligomers. We observed that the toxicity induced by the oligomeric strains was rescued in the presence of pharmacological inhibitors such as Dynamin and Heparin for dynamin-dependent and HSPGs-mediated pathways, respectively. This suggests that both strains were internalized via dynamin-dependent and HSPGs-mediated endocytosis. However, DA-modified α-Syn oligomers preferred dynamin-dependent endocytosis to HSPGs-mediated endocytosis. On the contrary, DHA-modified oligomers showed some preference for HSPGs-mediated pathway over dynamin-dependent pathway, although such difference was not significant.

Further investigation is required in this aspect to elucidate the intracellular fate of these oligomeric strains after being internalized.

CONCLUSION

Both DA and DHA are two extremely relevant biological conditions associated with PD pathogenesis. Overall, our findings provide useful insights into the role of biological inducers resulting in oligomeric α-Syn strains that might occur in the pathological condition.

CHAPTER 3

FUNCTIONAL HETEROGENEITY AMONG POLYMORPHIC ALPHA-SYNUCLEIN OLIGOMERS DERIVED FROM SYNUCLEINOPATHIES

ABSTRACT

Synucleinopathies comprise a diverse group of neurodegenerative disorders that are pathologically characterized by α-Synuclein (α-Syn) aggregates such as Lewy bodies (LBs) and Lewy neurites (LNs). The formation of α-Syn oligomers is considered as a critical event in the disease pathogenesis preceding inclusion formation. Little is known about the structural and functional heterogeneity among α-Syn oligomers occurring in different diseases, which perhaps may play roles in the disease phenotypes. Here, we have characterized brain-derived α-Syn oligomers (BDSOs) isolated from Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and Parkinson's disease (PD) by utilizing the techniques that were used to establish in vitro generated α-Syn oligomeric strains. We observed that the BDSOs displayed considerable differences in their morphologies and stability to proteolysis by proteinase K enzyme digestion. We noticed that upon exogenously application of these BDSOs to the primary cortical neurons, their accumulation pattern inside the cells is different. The three BDSOs also bound to heparan sulfate proteoglycans on the cell surface of soma and/or in the processes to different degrees. More notably, here we have also studied a gap junction protein, Connexin50 (Cx50), which bound to the BDSOs at different degrees, suggesting its involvement in the uptake of oligomers in the cells. To the best of our knowledge, this is the first study

showing functional heterogeneity among disease relevant α-Syn oligomers and the occurrence of Cx50 in the neuronal cells, suggesting its involvement in the uptake of pathogenic α-Syn oligomers.

BACKGROUND

Synucleinopathies include a diverse group of neurodegenerative disorders that are pathologically characterized by the presence of Lewy bodies (LBs) and Lewy neurites (LNs) comprised of α-Syn protein in neurons and glial cells (Spillantini and Goedert 2000). The three main synucleinopathies are Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (Marti, Tolosa et al. 2003). PD is the second most common form of dementia affecting almost 4 million people world-wide (Spillantini and Goedert 2000, Bovolenta, de Azevedo Silva et al. 2017). The incidence of PD is more pronounced among individuals of 60 years of age or above (Tysnes and Storstein 2017). DLB is one of the common forms of dementia and the second most commonly occuring synucleinopathy. The prevalence of DLB is over 1.3 millions in the United States accounting for almost 5% of all dementias in aged population (Hogan, Fiest et al. 2016). Alzheimer's disease (AD) is the most common cause of dementia and its incidence is almost 5.8 millions in the United States. AD is commonly viewed by the presence of aggregated hyperphosphorylated tau into neurofibrillary tangles (NFTs) and senile plaques of amyloid β protein. Intriguingly, LBs and LNs have been abundantly observed in the AD patients (Koehler, Stransky et al. 2013, Mattsson, Insel et al. 2013), thus categorizing AD as a synucleinopathy (Uchikado, Lin et al. 2006). Additionally, α-Syn protein was detected in the cerebrospinal fluid (CSF) of patients with AD and mild cognitive impairment (MCI) (Korff, Liu et al. 2013). In a transgenic mouse model

expressing amyloid precursor protein, α-Syn protein was detected in the amyloid plaques (Yang, Ueda et al. 2000).

It is well established that protein misfolding and pathogenic aggregation leading to abnormal accumulation is the key event of neurodegeneration (Ross and Poirier 2005). However, the mechanistic details of abnormal aggregation remains elusive. The formation of α-Syn oligomers is a critical event in the pathogenesis of PD and DLB preceding inclusion formation. Oligomers of α-Syn have been detected at an increased level in the postmortem brain tissues, in the blood plasma and CSF of PD and DLB patients (El-Agnaf, Salem et al. 2006, Park, Cheon et al. 2011, Mollenhauer, Trautmann et al. 2012, Sengupta, Guerrero-Munoz et al. 2015, Majbour, Vaikath et al. 2016). Although several mechanisms regarding α-Syn oligomers toxicity have been put forward, the exact mechanism is still undetermined. One of the challenges facing any conclusive outcome in the amyloid field is the profound heterogeneity among protein aggregates and α-Syn oligomers are not the exception. Different conditions and cofactors have been shown to cause different conformational and aggregation states of α-Syn (Breydo, Wu et al. 2012). Furthermore, different oligomers formed under different conditions showed variabilities in their toxic effects and seeding potencies (Danzer, Haasen et al. 2007)

The presence of α-Syn oligomeric assemblies in the extracellular body fluids such as blood plasma and CSF indicate their release from the affected brain regions and their subsequent spreading between cells. In cell culture, exocytosis via exosomes has been implicated for spreading of α-Syn (Emmanouilidou, Melachroinou et al. 2010). Also, the release of α-Syn oligomers via exosomes has been shown as an alternate pathway upon failure of autophagosomal clearance pathway (Danzer, Haasen et al. 2007). Additionally,

inoculation of brain extracts from patients with synucleinopathies into mice, caused the spreading of aggregated α-Syn among anatomically interconnected brain regions (Kordower, Dodiya et al. 2011, Masuda-Suzukake, Nonaka et al. 2013, Prusiner, Woerman et al. 2015). Several membrane-bound receptors such as heparan sulfate proteoglycan (HSPG), clathrin, cavoelin, lymphocyte-activation gene 3 (LAG3) and Fcγ receptor IIB (FcγRIIB) have been investigated to decipher the mechanism of cell-to-cell propagation of pathogenic α-Syn aggregates (Mao, Ou et al. 2016, Rodriguez, Marano et al. 2018). Both fibrillary α-Syn and exosome-associated oligomeric α-Syn were shown to be internalized in cells via HSPGs (Holmes, Furman et al. 2014, Delenclos, Trendafilova et al. 2017).

Connexin (Cx) proteins are part of the gap junctions maintaining cell-to-cell communications. These proteins regulate the exchange of molecules between cells by forming connecting bridges, known as gap junctions (Sohl and Willecke 2004). They are expressed in the brain, eye, heart, liver and in the smooth and endothelial muscles present in the blood vessels. Cx proteins play important functions in the CNS, where they are expressed in the neurons, astrocytes and endothelial cells (Xie, Cui et al. 2015). Cx32 and Cx43 are the two abundantly expressed Cx proteins in the brain. In a recent study, Cx32 has been shown to bind to α-Syn oligomers facilitating their uptake in the cells (Reyes, Sackmann et al. 2019). Both Cx43 and Cx30 were reported in an AD mouse model close to Aβ plaques (Xie, Cui et al. 2015). It has been shown in rotenone induced rat model that phosphorylated forms of Cx43 were increased in the astrocytes in basal ganglia. Such region-specific susceptibility of astrocytes to increased Cx43 has been suggested as a possible link to the damage of dopaminergic neurons in basal ganglia (Kawasaki, Hayashi et al. 2009). Upregulated expression of Cx43 in the astrocytes has been observed following

brain injury in rodent model (Cronin, Anderson et al. 2008). Another Cx protein, Cx36 is also associated with injury-mediated neuronal death, where reduced expression of this protein was shown to have neuroprotective effect (Paschon, Higa et al. 2012). One such gap junction protein, Cx50 is also expressed in the CNS. Although studies indicate the importance of Cx50 proteins present in the hemichannels of lens cells, there is almost no study indicating its association with α-Syn or any other amyloids in brain pathologies. The gene for Cx50 is located in 1q21-q22 chromosome, which has been shown as a susceptible gene in schizophrenia (Ni, Valente et al. 2007). To the best of our knowledge, this is the only study where Cx50 has been related to any brain illness.

Here, we have utilized the techniques that were used to establish recombinant α-Syn oligomeric strains and characterized brain-derived α-Syn oligomers (BDSOs). These BDSOs isolated from AD, DLB and PD brain tissues were characterized for their polymorphic nature. Since α-Syn oligomers are found in the CSF of Synucleinopathies patients, it suggests that the oligomers are stable in the CSF and can be detected by immunological methods. Therefore, we have used a preparation of recombinant α-Syn oligomers prepared in artificial CSF (aCSF) to compare the BDSOs. We have explored if the disease-relevant α-Syn oligomers can be endocytosed via HSPG-mediated pathway. Additionally, we have shown the presence of the gap junction protein Cx50 in neurons and in human brain tissues. We observed that different BDSOs bind with Cx50 at different degrees. Collectively, our results indicate that Cx50 associates with α-Syn protein in neurons and human brain tissues, suggesting that it could be involved in the uptake of α-Syn oligomers in cells. To the best of our knowledge this is the first study suggesting a potential involvement of Cx50 in the internalization and hence propagation of pathogenic

α-Syn oligomers. Further investigation is needed to gain more understanding about the role of Cx50 in human brain and also its association with α-Syn protein.

METHODS

Aggregation of α-Syn protein using aCSF

Artificial CSF solution was prepared by mixing 119 mM sodium chloride (NaCl), 26.2 mM sodium bicarbonate ($NaHCO_3$), 2.5 mM potassium chloride (KCl), 1 mM monosodium phosphate (NaH_2PO_4), 1.3 mM magnesium chloride ($MgCl_2$) and 10 mM glucose, followed by incubating the solution in a CO_2 Incubator (5% CO_2/95% $O_{2)}$ for 10-15 minutes. To this solution, 2.5 mM calcium chloride ($CaCl_2$) was added and filtered using a 0.22 μm filter and stored at 4 ^{0}C. Re-lyophilized α-Syn protein (0.6 mg) was dissolved in 1 mL of aCSF solution by stirring at 37 ^{0}C for 24 hours.

Preparation of brain extracts

Frozen frontal cortical brain tissue from Parkinson's disease, DLB, and age-matched control subjects were received in blocks from Oregon Health & Science University and the University of Maryland. Freshly prepared ice-cold PBS with protease inhibitor cocktail (Cat. # 11836145001, Roche Diagnostics, IN, USA) was added to each sample at a dilution of brain weight:buffer as 1:3 (w/v) and homogenized. The samples were then centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were collected. Brain homogenates (supernatants) were quantified using bicinchoninic acid protein assay (Micro BCA Kit, Pierce), aliquoted and stored at -80°C until use.

Isolation of α-Syn oligomers from Synucleinopathy brain tissues (BDSOs)

Immunoprecipitation of α-Syn aggregates was performed from the brain extracts of patients with synucleinopathies. Co-Immunoprecipitation Kit (Thermo Scientific) was used following manufacturer's guidelines. Briefly, amine-reactive resin was coupled with affinity-purified 4D6 antibody followed by incubating with brain extracts. Next, unbound proteins were washed off from the beads. Bound proteins were eluted in 0.1 M glycine (pH 2.8), adjusting the final pH to 7.0 by adding 1 M Tris-HCl (pH 8). Isolated fractions were subjected to buffer exchange and finally collected in sterile PBS. Immunoprecipitated α-Syn were then centrifuged in a microcon centrifugal filter device with 25 kDa molecular weight cut-off (Millipore) at 14,000 x g for 25 min at 4°C. The fractions of α-Syn that were retained in the filter device contained α-Syn oligomers. These oligomeric fractions were quantified using bicinchoninic acid protein assay (Micro BCA kit, Pierce). Fractions were then aliquoted and stored at -80°C. Oligomeric α-Syn isolated from brain tissues (BDSOs) were characterized thoroughly as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2012, Sengupta, Guerrero-Munoz et al. 2015).

Isolation of Cx50 from brain tissues

Immunoprecipitation of Cx50 was performed from PBS soluble fraction of brain homogenate from PD brain tissue. The anti-Cx50 antibody was used to pull down the protein following the above mentioned protocol.

Western blot Analysis

Oligomeric α-Syn preparations (1-2 µg) as well as well as brain homogenates (~ 15 µg) were loaded on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked

with 10% nonfat dry milk at 4°C overnight. Membranes were then probed with primary antibodies, LB509 (1:2000), anti-Cx50 (1:1000) and HRP-conjugated β-actin (1:6000) antibodies diluted in 5% nonfat dry milk for 1 h at RT. HRP-conjugated anti-mouse IgG (1:6000, GE Healthcare) and HRP-conjugated anti-mouse IgM were used to detect LB509 and Cx50 immunoreactivity, respectively. ECL plus (GE Healthcare) was used to visualize the bands. Densitometric analysis was performed using ImageJ software (National Institutes of Health).

Dot blot

Approximately 1.2 µl of anti-Cx50- and 4D6-immunoprecipitated materials were blotted onto nitrocellulose membrane. After overnight drying, dot blot strips were blocked with 10% nonfat dry milk for 1 h at RT. Membrane strips were then probed with primary antibody anti-Cx50 (1:1000) diluted in 5% nonfat dry milk for 1 h at RT. HRP-conjugated anti-mouse IgM was used to detect Cx50 immunoreactivity. ECL plus (GE Healthcare) was used to visualize the signal.

Atomic Force Microscopy

Atomic force microscopy was performed using 3 µl of each BDSO sample to study the morphologies of the isolated oligomers as described in Chapter 2.

Bis-ANS and Thioflavin T Fluorescence Assays

Binding of bis-ANS and Th T extrinsic fluorescent dyes with BDSOs have been performed as described in Chapter 2.

Immunofluorescence

Immunofluorescence labeling was performed with frozen sections of PD, DLB, AD and control brains. Frozen sections were fixed in chilled methanol followed by blocking in goat serum for 1 hour. Sections were then incubated in Cx50 antibody (1:500) overnight. The next day, sections were washed three times with PBS and then incubated with goat anti-mouse IgM Alexa-568 (1:700, Invitrogen) for 1 h. Sections were then washed three times with PBS and incubated overnight with 4D6 antibody (1:350) at 4°C. After washing three times with PBS (10 min each), sections were then incubated with goat anti-mouse IgG Alexa- 488 for 1 h. They were then washed, stained with DAPI (Vector Laboratories) and mounted in Vectashield mounting medium (Fluoromount-4',6-diamidino-2-phenylindole. The slides were imaged with a Keyence BZ-800 Microscope and analyzed using BZ-X Analyzer. A Nikon 40X and 100X oil immersion objectives was used for image acquisition. Images were taken from 3 different fields from 3 different sections per group using identical laser power, photomultiplier gain, and pinhole settings. Images were analyzed with ImageJ (National Institute of Health). One-way ANOVA followed by Tukey's post hoc was used to quantify the co-localized intensities.

Primary Cortical Neuron Culture

The C57BL/6N-Tg(Thy1-SNCA)15Mjff/J (Jackson Laboratory, Stock# 017682) were used for primary cortical neuron isolation. Animals were maintained in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. All required procedures were carried out as per the recommendations of Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for primary neuron isolation was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB). Primary cortical neuronal cells from

C57BL/6N-Tg (Thy1-SNCA)15Mjff/J mice were isolated and maintained as described in Chapter 2.

Primary Neurons Treatment, Immunostaining and Confocal Microscopy

Primary cortical neurons grown on coverslip in a 24-well plate were exposed to 0.25 µM BDSOs for 8 h. For vehicle treated group, 1 X PBS was added to the neuronal cells and incubated for the same time period. After 8 h of incubation, cells were washed 3 times with 1X PBS and fixed with 4% formaldehyde solution for 15 min at RT. Cells were then washed 3 times with 1 X PBS followed by permeabilizing with 0.25 % Triton X-100 in PBS for 10 min at RT. Cells were blocked in 5% goat serum for 30 min at RT and incubated with primary antibody, mouse LB509 (1:600) at 4°C overnight. Next day, cells were washed and incubated with secondary antibody Alexa fluor anti-mouse 568 (1:1000, Life Technologies) for 1 h at RT. Coverslips were incubated with either mouse anti-Cx50 (1:600) or rabbit anti-HSPG (1:600) at 4°C overnight. Next day, following 3 washes, coverslips were incubated with either Alexa fluor anti-mouse 488 or Alexa fluor anti-rabbit 488 (1:1000, Life Technologies) at RT for 1 h. For Following 3 washes, coverslips were incubated with either anti-MAP2 (1:1000) or anti-β-III tubulin (1:1000) at RT for 1.5 h. Following 3 washes, coverslips were incubated for either Alexa fluor anti-rabbit 305 or Alexa fluor anti-mouse 305 (1:1000, Life Technologies) at RT for 1 h. Coverslips were finally washed for 3 times and mounted with ProLong Diamond antifade mounting media without DAPI (Invitrogen).

Coverslips with all treatment conditions were imaged under Zeiss LSM 880 confocal microscope using 63x objective with 405 nm diode laser and argon laser 458/488/514nm. Z-stacks were built by capturing images from approximately 16-17 stacks

at 0.37-0.41 μm optimal thickness. Each treatment condition was performed in 3 independent experiment and were randomly imaged at five different regions of interest. All images were analyzed by ImageJ (NIH) software.

Statistical Analysis

All statistical analyses were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). All values were calculated as mean and standard deviation (SD). Data are presented from at least 3 replicates and from 3 independent experiments. For fluorescent correlation measurement of confocal microscopic images, immunofluoresce images of human brain tissues, bis-ANS and thioflavin T fluorescence, and quantification of Western blot bands, one-way ANOVA with Tukey's multiple comparisons test was performed. The number of experiments is mentioned in the figure legends.

RESULTS

Characterization of Disease-associated Brain-Derived α-Syn Oligomers (BDSOs)

Elevated oligomers of α-Syn have been detected from the soluble fractions extracted from cortices of brain tissues of PD and DLB patients. We have also previously established the isolation and characterization of oligomeric assemblies of α-Syn and tau from brain tissue in their most stable form (Sengupta, Guerrero-Munoz et al. 2015). Therefore, following our published protocol, immunoprecipitation of α-Syn aggregates

was performed using a generic α-Syn antibody, 4D6. To obtain all possible aggregates of α-Syn, a sequence specific antibody against of α-Syn has been chosen for

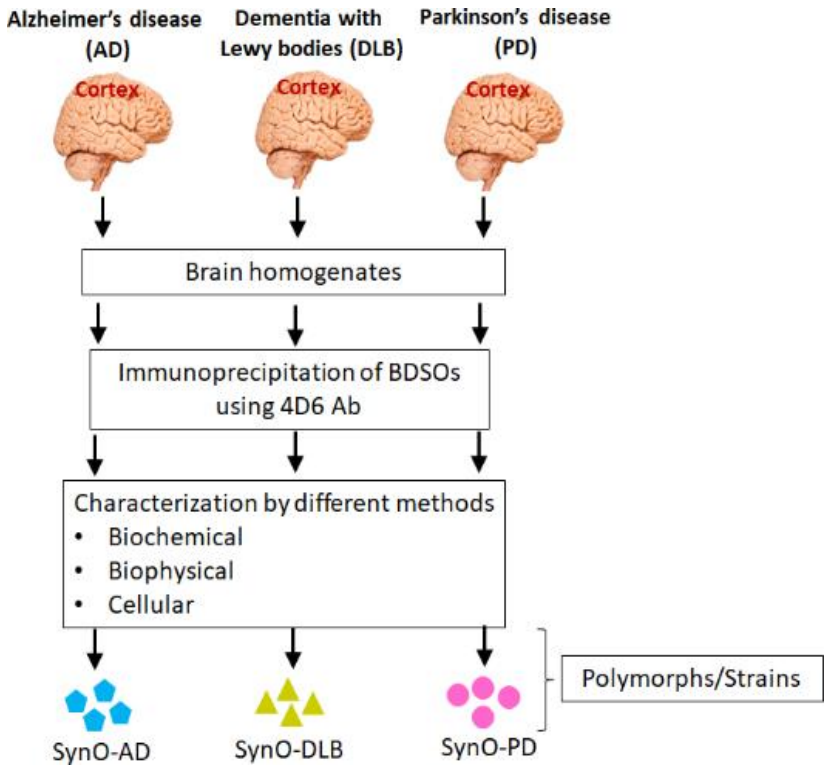

Figure 3. 1 Schematic representation of isolation and characterization of α-Syn oligomers derived from brain tissues of Synucleinopathies (BDSOs).
Cortical regions of AD, DLB and PD brain tissues were homogenized to obtain PBS-soluble fractions. Immunoprecipitation was performed using generic α-Syn antibody, 4D6 to isolate α-Syn. Isolated materials were filtered by size exclusion chromatography to obtain oligomeric assemblies, which were characterized by biochemical, biophysical and cellular techniques

immunoprecipitation. Isolated α-Syn aggregates were separated by size exclusion chromatography and oligomeric assemblies were obtained for all three pathologies and characterized via various biochemical and biophysical methods (**Figure 3.1**). Western blot analyses of BDSOs using LB509 antibody specific for human α-Syn protein showed dimers and oligomers of higher molecular weight (~ 250 kDa and above) (**Figure 3.2A**). Separately, we oligomerized recombinant α-Syn protein using artificial cerebrospinal fluid

(aCSF) and used as a control oligomeric preparation for the BDSOs. SynO-aCSF also showed higher aggregates, however, SynO-aCSF showed strong monomeric signal than

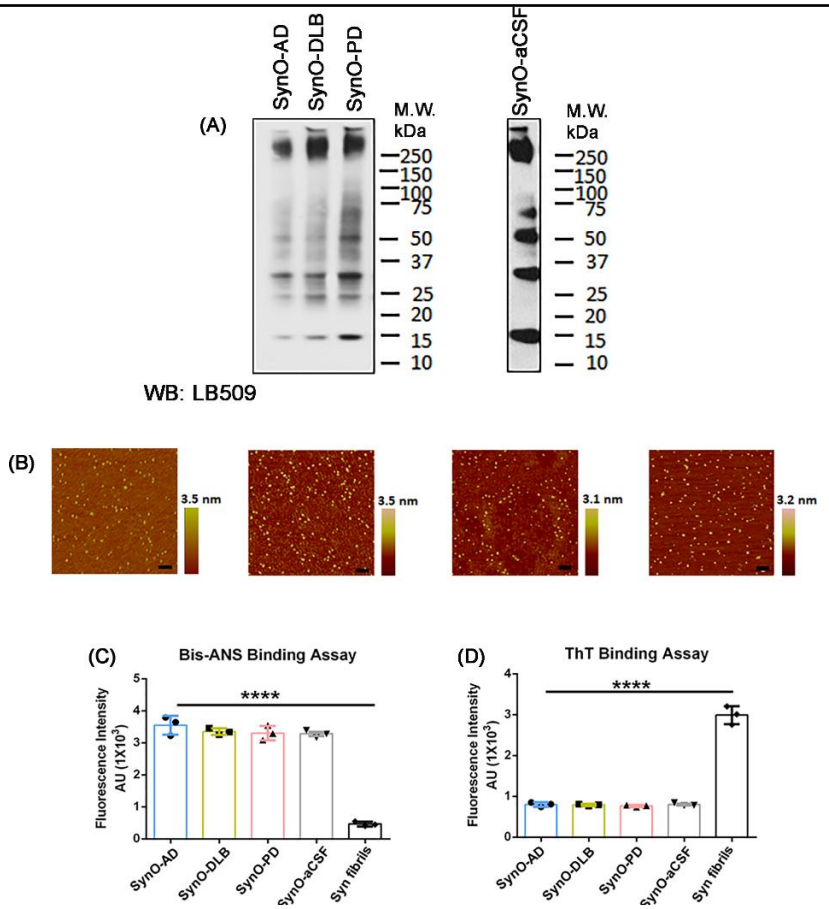

Figure 3.2 Biochemical characterization of brain-derived α-Syn oligomers (BDSOs).
(**A**) Representative Western blot images of three BDSOs: SynO-AD, SynO-DLB and SynO-PD immunolabeled with a generic α-Syn antibody, LB509. The BDSOs showed different orders of aggregates. Western blot images of recombinant α-Syn oligomer prepared in aCSF (SynO-aCSF) probed with LB509 antibody also showed oligomers with a more prominent monomeric signal. (**B**) Representative AFM images of BDSOs. All four oligomer preparations showed spherical structures. Scale bar 100 nm. (**C**) Fluorescence intensity of bis-ANS binding to α-Syn aggregates showed significantly strong binding to all the three BDSOs and SynO-aCSF compared to Syn fibrils. (**D**) Fluorescence intensity of ThT binding to α-Syn aggregates. ThT binding to all four α-Syn oligomer preparations is significantly less compared to Syn fibrils. Data are represented as mean and SEM from 3 independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparisons test; **** $p < 0.0001$.

the three BDSOs (**Figure 3.2A**). We studied the morphology of the brain-derived oligomers by AFM (**Figure 3.2B**). The brain-derived oligomers showed spherical structures under AFM as was also observed for SynO-aCSF preparation. To assess the hydrophobicity and aggregation state of the BDSOs, we performed fluorescence binding assays using bis-ANS and thioflavin T (ThT). All the BDSOs showed significantly increased binding with bis-ANS, which was comparable to the recombinant α-Syn oligomer, SynO-aCSF. As expected, fibrillar α-Syn showed less binding with bis-ANS (**Figure 3.2C**). On contrary, all four oligomers showed less affinity for ThT as compared to α-Syn fibrils (**Figure 3.2D**). These results indicate that the brain-derived oligomers exhibit exposed hydrophobic surfaces as of the control recombinant oligomer (SynO-aCSF).

Brain-derived α-Syn Oligomers have Differential Sensitivity to Proteinase K

After characterizing the three BDSOs, next we evaluated the qualitative conformational differences between them and their stability as oligomers. We measured their sensitivity for Proteinase K (PK) enzyme digestion, as we performed with in vitro α-Syn oligomeric polymorphs. PK digestion method had been discovered in classifying strains of prion fibrils, which has later been extended to identifying other amyloid strains, such as amyloid-β, α-Syn and tau fibrils (Guo, Covell et al. 2013) (Supattapone, Muramoto et al. 2001, Colby, Wain et al. 2010). We treated SynO-AD, SynO-DLB, SynO-PD and SynO-aCSF with increasing concentrations of PK enzyme (0, 1, 1.5 and 2 µg/mL) (**Figure 3.3)**. All the digested samples were then run in SDS-PAGE gel followed by silver staining to visualize the fragments. The pattern of fragments generated by PK digestion provides information on the stability of the oligomers, as well as its core. We observed different

patterns of fragmentation of these oligomers followed by PK digestion as highlighted inside boxes (**Figure 3.3**A). To visualize the differences among the patterns of PK-digested fragments more clearly, the bands from three molecular weights were quantified and

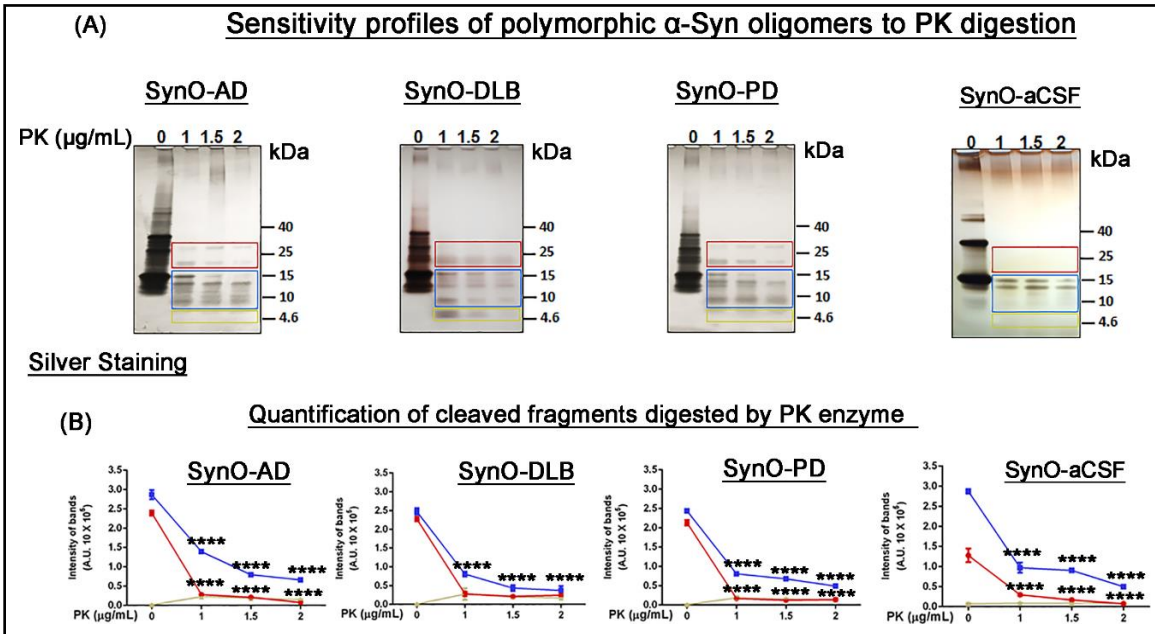

Figure 3.3 Proteolytic digestion profiles of brain-derived α-Syn oligomeric (BDSO) polymorphs.
(**A**) Silver staining images of BDSOs, SynO-AD, SynO-DLB, SynO-PD and SynO-aCSF digested with 1, 1.5 and 2 µg/mL Proteinase K (PK) enzyme. The pattern of fragments at three different ranges of molecular weights are highlighted in boxes: ~ 25 kDa (red box), ~ 10-15 kDa (blue box) and ~ 4.6 kDa (yellow box). (**B**) Densitometric quantification of the fragments digested by PK enzyme compared with the band from untreated sample (0 PK). The colors of the lines correspond to the fragments in each color box in (**A**). Each line represents mean and SEM calculated from three independent experiments. Statistical significance is calculated using one-way ANOVA with Tukey's multiple comparisons test. **** $p < 0.0001$.

compared with the undigested counterparts for all the three samples (0 PK) (**Figure 3.3B**). At ~25 kDa molecular weight (red box), Syn-AD and Syn-PD showed two fragments, whereas SynO-DLB showed a strong single band. SynO-aCSF did not show any band at this molecular weight (**Figure 3.3A, B; red box and red line**). SynO-AD and SynO-DLB showed three major bands with some weak bands at ~ 10-15 kDa molecular weight (blue box). But, the spatial distribution of bands in these two oligomeric samples were different.

On the other hand, SynO-PD showed four prominent bands with 1 µg/mL PK concentration at 10-15 kDa molecular weight, which reduced in intensity at 1.5 and 2 µg/mL PK concentrations. SynO-aCSF showed mostly two strong bands with similar intensities at both 1 and 1.5 µg/mL PK concentrations (**Figure 3.3A, B; blue box and blue line**). At ~ 4.6 kDa molecular weight (yellow box), SynO-AD and SynO-aCSF did not show any visible bands, however, SynO-DLB showed a prominent banding pattern that gradually decreased in its intensity with increasing concentration of PK enzyme (**Figure 3.3A, B; yellow box and yellow line**). SynO-PD showed a faint band at 4.6 kDa molecular weight. These data together indicate that the three BDSOs: SynO-AD, SynO-DLB and SynO-PD can be considered as three distinct strains of brain-derived α-Syn oligomers.

Exogenous BDSOs are localized in different cellular compartments upon intake by the primary neurons

Next, we determined to which extent the exogenous applied oligomeric species of BDSOs derived from AD, DLB and PD cases were localized in the recipient cells upon intake. Primary cortical neurons from transgenic mice overexpressing human α-Syn protein (Tg-hSyn) were exposed to well-characterized BDSOs and SynO-aCSF at 0.25 µM for 8 h. Cells were then immunostained with LB509 antibody for human α-Syn protein and anti-MAP2 antibody (microtubule-associated protein 2) to identify neuronal cells followed by confocal microscopic analyses (**Figure 3.4**). In neurons, MAP2 is mostly expressed in the dendrites and perikarya of the cells.

Since MAP2 is a perikaryal marker protein including nucleus, therefore, in absence of any nucleus-specific staining, we quantified the signal of punctate α-Syn oligomers in association with cell body excluding processes and nucleus as well as

separately in association with nucleus only. Confocal microscopic analyses of the oligomers treated cells revealed that oligomeric species of α-Syn derived from different

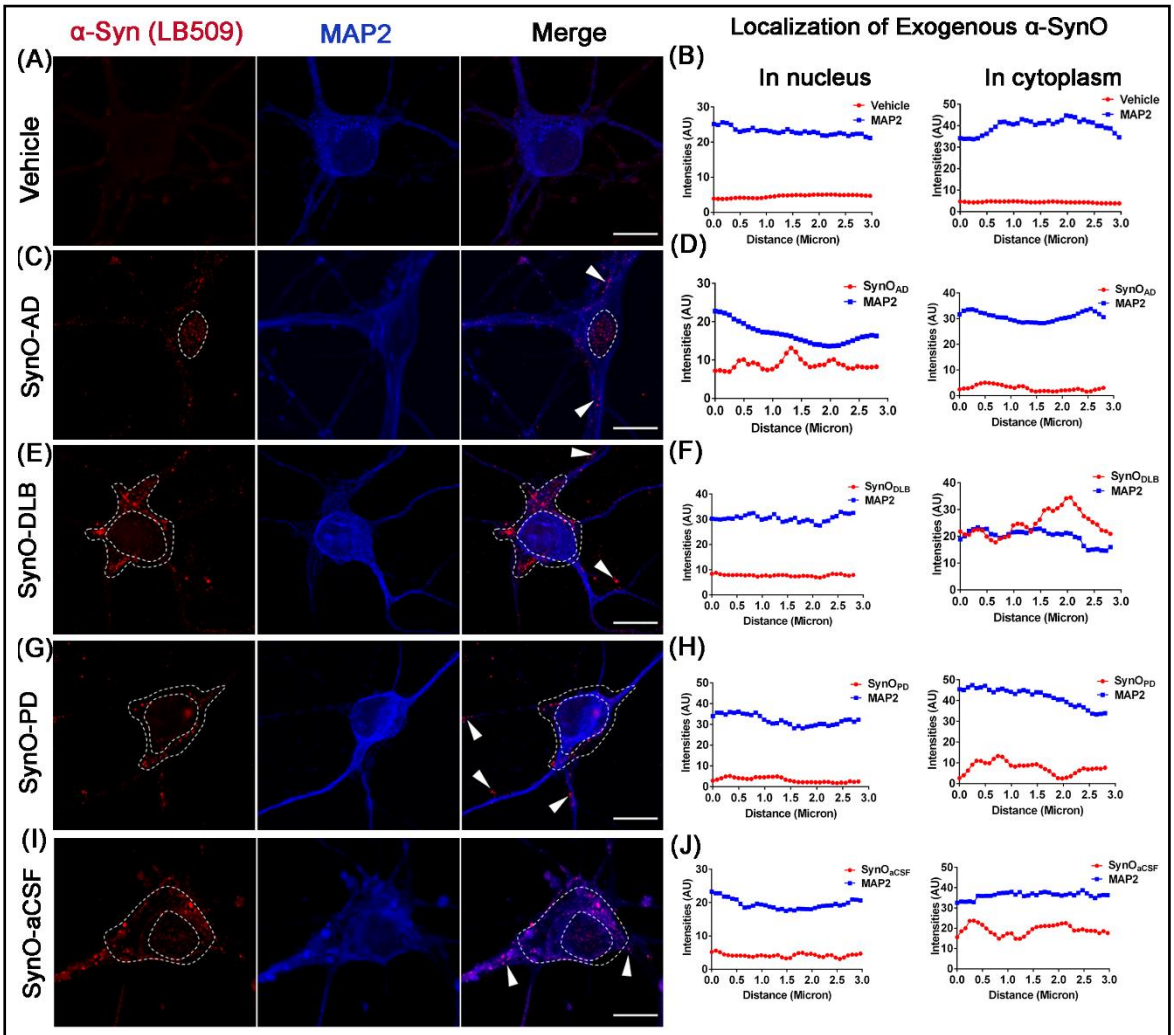

Figure 3.4 Patterns of BDSOs accumulated in primary cortical neurons.
Primary cortical neurons from Tg-hSyn mice embryos were exposed to three different BDSOs and SynO-aCSF followed by immunolabeling with LB509 for α-Syn oligomers and MAP2 as neuronal marker. (**A, B**) Vehicle treated cells are negative for α-Syn oligomeric signal. (**C, D**) SynO-AD primarily appeared to be accumulated in the nuclear region (white arrowheads). This was also evident from the plot profile of the nucleus showing close proximity between the intensities of SynO-AD and MAP2 signals, compared to the cell soma. (**E, F**) SynO-DLB were accumulated in the perikayral region along the cell soma (white arrowheads), also shown in the plot profile of cell soma as compared to nucleus. (**G, H**) SynO-PD also accumulated mostly along the cell soma (white arrowheads), however, the intensity observed for SynO-PD was less compared to SynO-DLB. (**I, J**) SynO-aCSF mostly showed accumulation in the cell soma (white arrowheads) than nuclear region. All the four types of α-Syn oligomers were also noticed along the cell processes. Scale bar 10 µm.

pathologies showed different patterns of accumulation. For each treatment group, representative image and its associated plot profiles (in nucleus and cell soma) are shown. In cells treated with AD brain-derived oligomers, the punctate signals of SynO-AD were mostly observed in the nucleus (dotted region) and to soma extent in the cell soma and processes (**Figure 3.4C**; white arrowheads). Plot profiles of oligomeric signal in association with nuclear signal showed overlapping intensities, indicating the primary localization of these oligomers in the nucleus (**Figure 3.4D**). On the contrary, DLB brain-derived α-Syn oligomers, SynO-DLB showed accumulation in the cell soma (dotted region) as compared to the nucleus which was also observed from the corresponding plot profiles (**Figure 3.4E**). The intensity of oligomeric signal greatly overlapped with signal intensity of the cell soma with a comparatively weak signal in the nucleus (**Figure 3.4F**). In SynO-PD treated group, accumulated oligomeric signals were mostly observed in the cell soma (dotted region) with very negligible amount in the nucleus (**Figure 3.4G, H**). Cells treated with SynO-aCSF, oligomers were noted in the cell body at higher intensity than the nucleus, while in the vehicle treated control group, and we observed a flat line indicating no oligomeric signal (**Figure 3.4I, J**). In all the treated groups, oligomers were also noted in the processes (white arrowheads). As expected, vehicle treated cells neither did any oligomeric signal nor colocalization with MAP2 signal (**Figure 3.4A, B).** These results suggest that the oligomers derived from different pathologies were internalized through the cell body and perhaps, through the processes and they accumulated inside the cells at different degrees.

Brain-Derived α-Syn Oligomers Colocalize with HSPG receptors in Primary Neurons

In our previous experiments with in vitro α-Syn oligomers induced by DA and DHA, we

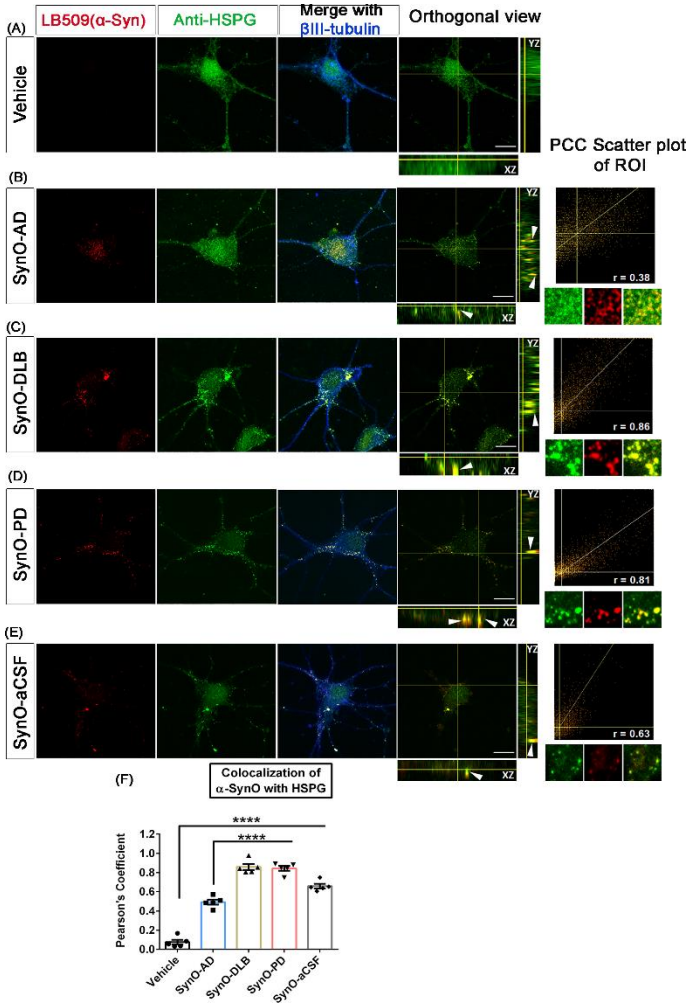

Figure 3.5 Colocalization of BDSOs with HSPGs in primary cortical neurons from Tg-hSyn mice embryos.

Primary cortical neurons were exposed to vehicle and 0.25 μM BDSOs and SynO-aCSF as well as with vehicle for 8 h (**A-E**). Cells were immunostained with LB509 for α-Syn (red), anti-HSPG antibody (green) for heparan sulfate and βIII-tubulin for neuronal marker (blue). Vehicle treatment (**A**) did not show any signal for α-Syn oligomers and hence no colocalization was noticed between oligomers and HSPG, although HSPG expression was observed. SynO-AD (**B**), SynO-DLB (**C**), SynO-PD (**D**) and SynO-aCSF (**E**) colocalized with HSPG. Representative orthogonal view for each treatment confirmed the colocalization between BDSOs and HSPG. Pearson's Correlation coefficient (PCC) scatter plot of region of interest (ROI, as shown below the PCC scatter plot) showed a strong correlation of SynO-DLB and SynO-PD with HSPG (Pearson's Correlation coefficient (R) value as 0.86 and 0.81 respectively) compared to SynO-AD with HSPG (R=0.53). SynO-aCSF showed colocalization with HSPG with R value of 0.63. (**F**) Quantitative analysis of colocalized intensities from α-SynO and HSPG from 5 regions of interest (ROIs) from 3 different cells. One-way ANOVA with Tukey's multiple comparison test;**** $p < 0.0001$; Scale bar 10

observed that both oligomeric strains can be internalized by HSPG-mediated and dynamin-mediated endocytic pathways (Chapter 2: **Figure 2.9**). Based on the observation that the brain-derived α-Syn oligomers are uptaken by the primary cortical neurons, we next investigated whether HSPG-mediated endocytic pathway is involved in the uptake of the BDSOs. Additionally, we sought to investigate if HSPG-mediated α-Syn oligomers uptake is disease-specific or not. A previous study had shown that recombinant α-Syn fibrils were taken up by rat neuroblastoma B103 cells at 4h and 24h of incubation (Ihse, Yamakado et al. 2017). In this study, primary cortical neurons from Tg-hSyn embryos were exposed to BDSOs at 0.25 µM for 8 h and immunostained with LB509 (α-Syn), anti-heparan sulfate antibody (HSPG) and βIII-tubulin (neuronal marker) antibody. Using confocal microscopy, we visualized that almost all BDSOs directly bind to the HSPG, but to different degrees. As observed before that the SynO-AD appeared to be localized in the nucleus, colocalization between SynO-AD and HSPG was mainly centralized in the nuclear region, also shown in orthogonal view (white arrowheads) (**Figure 3.5B**). To evaluate the extent of BDSOs and HSPG colocalization, Pearson Correlation coefficient (r) was calculated. Representative PCC scatter plot of a region of interest (ROI: ~ 4 x 4 µm) indicate the colocalization of SynO-AD and HSPG, but to lesser extent (**Figure 3.5B**). In contrast, both SynO-DLB and SynO-PD showed strong association with HSPG as observed around the cell soma and the processes (**Figure 3.5C, D)**. The difference in colocalization between SynO-AD with SynO-PD and SynO-DLB was statistically significant (**Figure 3.5F).** Representative orthogonal views demonstrated the colocalization between the two oligomers and HSPG, which were also highlighted by the PCC scatter plots. The Pearson correlation coefficient (r) of 0.86 and 0.81 also confirmed

the strong association of SynO-DLB and SynO-PD with HSPG, respectively (**Figure 3.5C, D**). SynO-aCSF showed moderate association with HSPG as compared to SynO-DLB and SynO-PD with a correlation coefficient of 0.63 (**Figure 3.5F**). Collectively, these results indicate the involvement of HSPG-mediated endocytic pathway in uptake of different BDSOs. However, our data also imply that the degree of binding of different BDSOs with HSPG varies. Analysis of colocalization was performed based on Pearson's correlation coefficient (r) (**Figure 3.5F**).

Uptake of Disease-associated α-Syn Oligomers via Gap Junction Protein Cx50

As gap junction proteins play important roles in the transfer of molecules between cells, they might possess great role in the transfer of pathogenic oligomers. We sought to investigate whether gap junction protein Cx50 is involved in the binding and uptake of α-Syn oligomers in neurons. The three BDSOs: SynO-AD, SynO-DLB, SynO-PD and SynO-aCSF were exogenously added to the Tg-hSyn primary cortical neuros at 0.25 µM concentration and the cells were incubated for 8 h. The optimum concentration of oligomers and time of incubation were determined empirically. Triple immunostaining with anti-Cx50 antibody (green) and LB509 antibody (red) of the treated neuronal cells was performed to visualize the localization of exogenously applied oligomers (BDSOs) with Cx50. Anti MAP2 antibody (blue) was used as neuronal marker. Confocal microscopic analysis showed SynO-AD mostly colocalized with Cx50 both in the nucleus, which was also observed by orthogonal view and to some extent in the processes (**Figures 3.6B and 3.7A**). In SynO-DLB treated neurons, a more prominent colocalization between oligomers and Cx50 was observed around the cell soma, in the perikarya and in the processes (**Figure 3.6C)**. A clear association of α-Syn oligomers with Cx50 around the cell

soma in orthogonal view indicate that the oligomers are bound to this gap junction protein

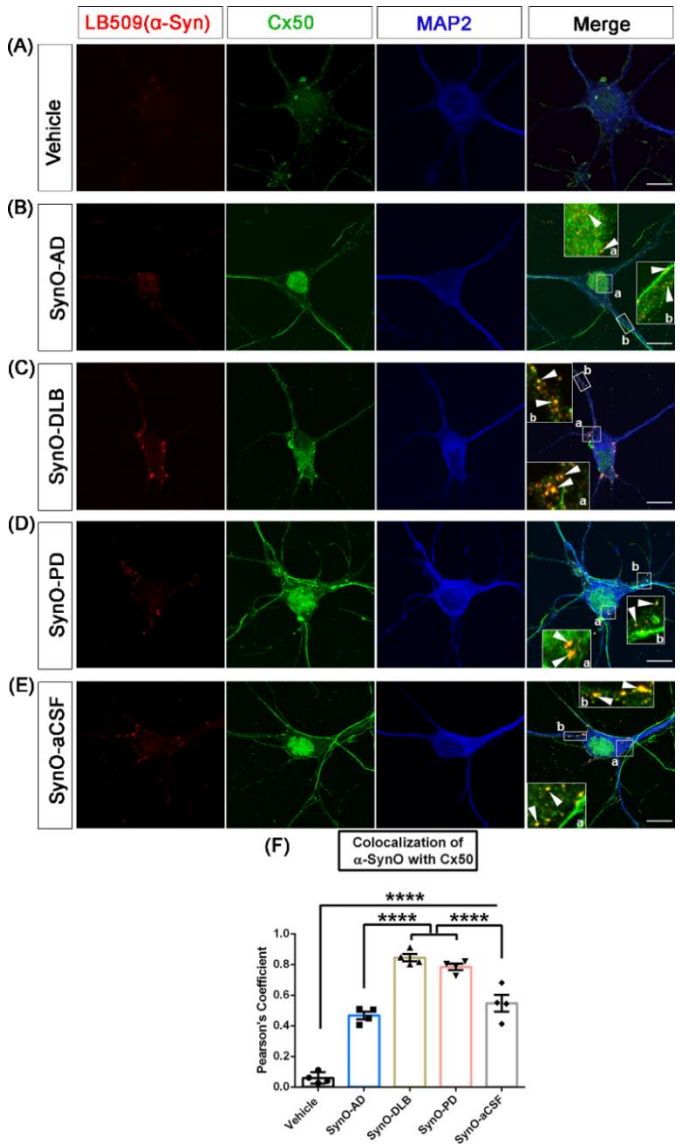

Figure 3.6 Colocalization of BDSOs and SynO-aCSF with gap junction protein Cx50 in primary cortical neurons from Tg-hSyn mice embryos.
Primary cortical neurons were exposed to 0.25 μM BDSOs and SynO-aCSF as well as with vehicle for 8 h. **(A)** Vehicle treated neurons showed expression of Cx50. (**B**) In SynO-AD treated cells, oligomers mostly colocalized with Cx50 in the nuclear region (a) and processes (b). (**C, D**) Strong colocalization of SynO-DLB and SynO-PD with Cx50 was noticed along the cell soma (a) and processes (b). (**E**) SynO-aCSF also showed considerable colocaliztion with Cx50. (F) Analysis of Pearson's correlation coefficient showed significantly increased colocalization of SynO-DLB and SynO-PD compared to SynO-AD and SynO-aCSF. Calculations were performed from five different regions of interest (ROIs) in triplicates. One-way ANOVA with Tukey's multiple comparisons test; **** $p < 0.000$. Scale bar 10 μm.

(**Figure 3.7B)**. In the SynO-PD treated neurons, oligomers mostly colocalized with Cx50 in the cell body and in the processes (**Figure 3.6D)**. Orthogonal view indicated that in this group the oligomers were mostly bound to Cx50 along the edges of the cell soma (**Figure**

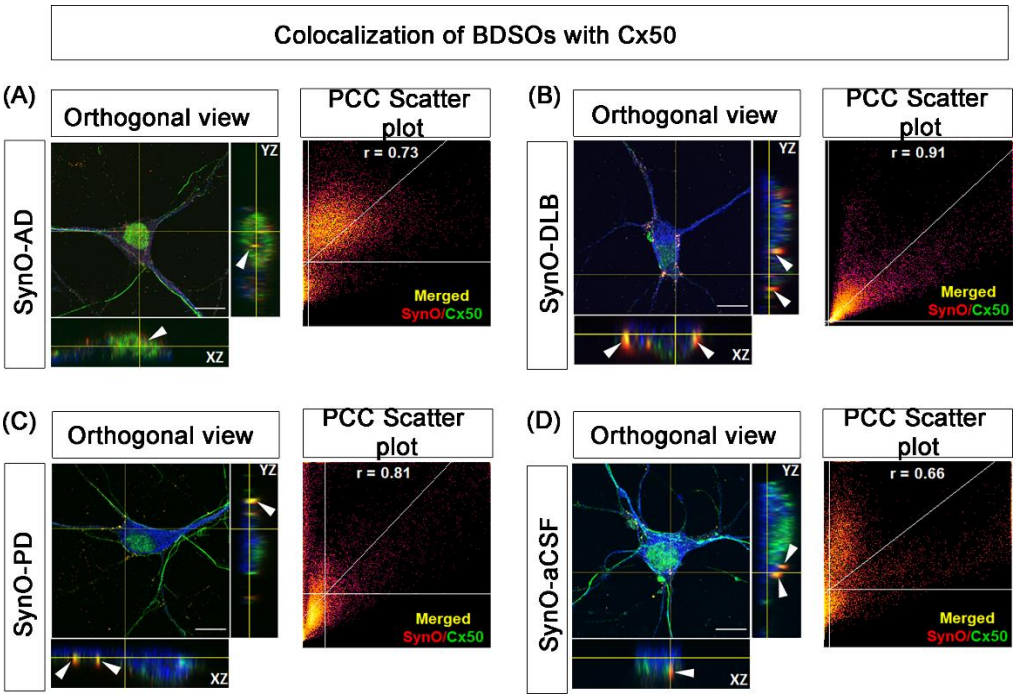

Figure 3.7 Correlation analysis of colocalized α-Syn oligomers with gap junction protein Cx50.
In primary cortical neurons from transgenic animals expressing human α-Syn, α-Syn oligomers colocalized significantly with Cx50. Orthogonal views and Pearson correlation coefficient scatter plots Cx50 colocalizing with SynO-AD, r = 0.73 (A), SynO-DLB, r = 0.91 (B), SynO-PD, r = 0.81 (C) and SynO-aCSF, r =0.66 (D), showing strongest association of SynO-DLB and SynO-PD with Cx50. Scale bar 10 µm.

3.7C). Neurons treated with SynO-aCSF showed accumulation in the cell body and in the processes also showed in the orthogonal view (**Figures 3.6E and 3.7D)**. As expected, in the vehicle treated control group, there was no oligomeric signal. Surprisingly, in this control group even the expression of Cx50 protein was different than the oligomers treated groups (**Figure 3.6A)**. Analysis of Pearson's correlation coefficient showed significantly increased colocalization of SynO-DLB and SynO-PD compared to SynO-AD and SynO-aCSF (**Figure 3.6F)**. These observations suggest that α-Syn oligomers can be taken up in

cell body as well in the processes via binding with Cx50. They also indicate the increased expression of Cx50 upon oligomer treatment as compared to a baseline expression in the vehicle treated group.

Occurrence and Expression of Cx50 in Human Brain Tissues

Studies have suggested a putative role of astrocytic Cx43 in PD pathogenesis (Xie, Cui et al. 2015). Having observed that the Cx50 is expressed in the mouse primary cortical neurons, we next investigated whether Cx50 would be expressed in human brain tissues. Therefore, we analyzed cortical sections of brain tissues from AD, DLB and PD patients as well as age-matched control subjects by immunostaining with LB509 (α-Syn) and anti-Cx50 antibodies. In our previously published work, we have demonstrated by immunofluorescence and biochemical analyses that α-Syn oligomers are present at an elevated levels on the brain tissues of PD and DLB patients (Sengupta, Guerrero-Munoz et al. 2015). As expected, immunofluorescence analysis with LB509 antibody showed the presence of α- Syn (in green) protein in all the pathologies (**Figure 3.8A, B**). Additionally, immunofluorescence of the brain sections with anti-Cx50 antibody clearly showed the presence of Cx50 (in red) in the cases studied. The appearance of Cx50 was mostly punctate around the nuclei (in blue). Notably, in both AD, DLB and PD cases, α-Syn and Cx50 colocalized. To further assess the in vivo occurrence of Cx50, we biochemically analyzed DLB, PD and AD cases, as well as age-matched controls (DLB, PD, AD, Ctrl; N=2). Western blot analysis of PBS-soluble brain homogenates (~ 15 µg of total protein) immunolabeled with anti-Cx50 antibody showed the presence of Cx50 in all the brain homogenates, indicating its occurrence in human brain tissues (**Figure 3.8C, D**).

Surprisingly, we observed double bands in DLB, PD and control brain homogenates, which

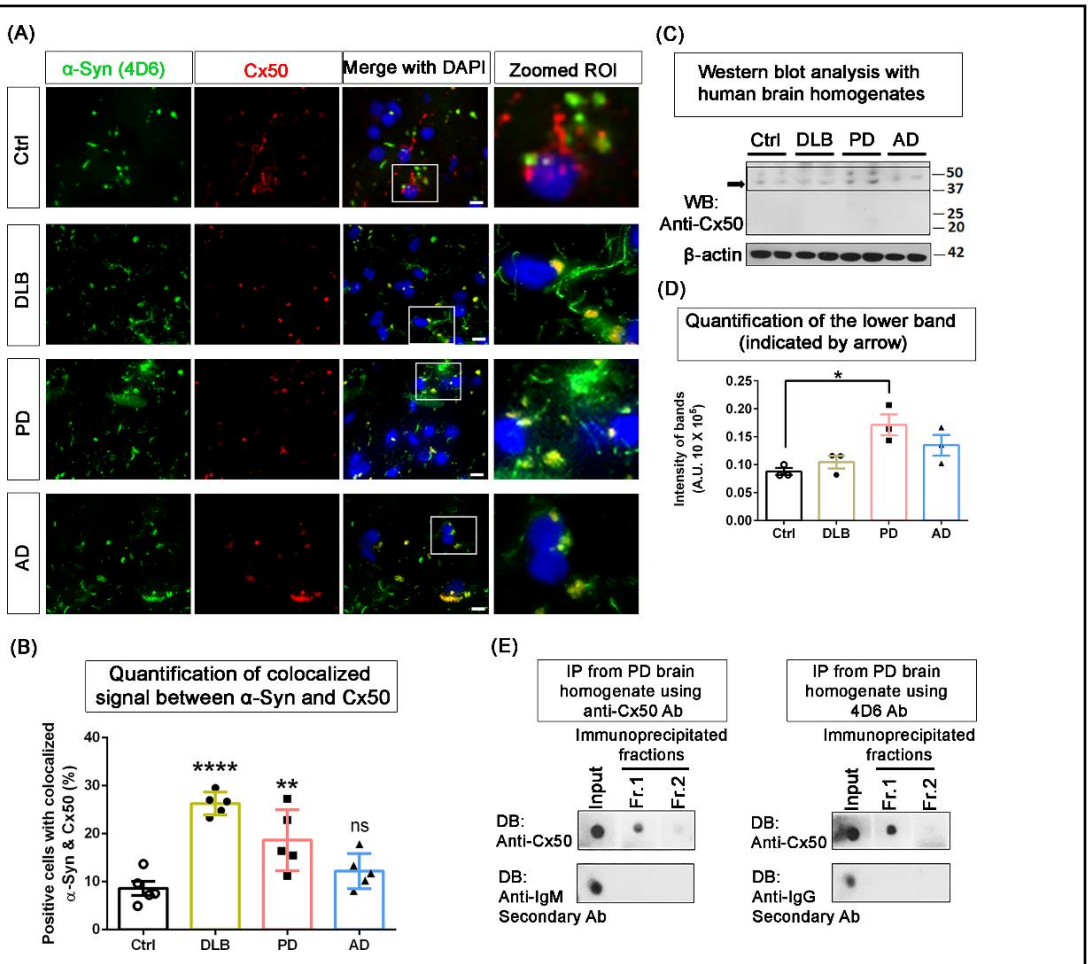

Figure 3.8 Detection of Cx50 in human brains tissues.
(**A**) Representative Western blot image of PBS soluble fractions of PD, DLB, AD and age-matched control brain homogenates probed with anti-Cx50 antibody showed the presence of Cx50 in the brain tissues. β-actin was used as loading control. (**B**) Densitometric quantification of the band at ~50 kDa normalized with β-actin signal (indicated by arrow in (**A**)). (**C**) Representative dot blot image of immunoprecipitated Cx50 from PBS soluble fraction of PD brain homogenate immunolabeled with anti-Cx50 antibody confirmed the presence of this protein in human brain tissue. Probing with secondary antibody only (anti-mouse IgM) did not show any signal in the immunoprecipitated sample. Representative dot blot image of immunoprecipitated α-Syn using 4D6 antibody from PBS soluble fraction of PD brain homogenate immunolabeled with anti-Cx50 antibody also detected Cx50 protein. Probing with secondary antibody only (anti-mouse IgG) did not show any signal in the 4D6-immunoprecipitated sample. (**D**) Representative epifluorescence images of cortical sections from PD, DLB, AD and age-matched control brain tissues immunostained with anti-Cx50 (red) and 4D6 (green). Zoomed region of interest (ROI) showed partial colocation between α-Syn and Cx50. The quantification is represented as mean and SEM from two cases (N=2) performed in two independent experiments. One-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$. Scale bar 10 µm.

was not found in AD cases. It is reported that Cx proteins can form isoforms or produce fragments (Esseltine and Laird 2016). Thus, the second band observed here could be an isoform of Cx50 or might be resulted from the phosphorylation of Cx50 protein. In a rat model of TBI, increased level of phosphorylated Cx43 has been documented following brain injury (Chen, Guo et al. 2018). Phosphorylation of proteins has been associated with several neurodegenerative diseases. Further studies will be required to dissect out more clearly if there are isoforms or phosphorylation of Cx50 present in these brain tissues or not. To further confirm our observation of Cx50 occurring in the human brain tissues, we immunoprecipitated Cx50 from PD brain homogenate since the signal for this pathology was the most strong in Western blot. Representative dot blot images of Cx50 immunoprecipitated material labeled with anti-Cx50 antibody confirmed the presence of this connexin protein in human brain tissues (**Figure 3.8E**). Probing with secondary antibodies alone did not show any signals in the immunoprecipitated material, indicating the successful pulling down of Cx50 protein (**Figure 3.8E**). Furthermore, probing with 4D6 antibody showed signal in the anti-Cx50 immunoprecipitated material. This indicates that Cx50 and α-Syn might be present together.

DISCUSSION

Synucleinopathies are comprised of several neurodegenerative diseases that manifest a common pathophysiology, the presence of α-Syn protein aggregates. Heterogeneity among in vivo protein aggregates pose a big challenge to study them in native forms and α-Syn oligomers are not the exception. Here, we aimed to study the α-Syn oligomers that occur in diseases (BDSOs), specifically in Synucleinopathies more in depth. Considering that the

oligomers of amyloid proteins act as the major entities causing disease pathogenesis, to the best of our knowledge, this is the first study demonstrating the occurrence of polymorphic α-Syn oligomers in AD, DLB and PD pathologies.

Large α-Syn oligomers preferentially bind to SNARE protein synaptobrevin-2 and inhibit the SNARE complex formation, inhibiting vesicle docking and neurotransmitter release (Choi, Choi et al. 2013). Small diffused granular α-Syn aggregates have enormously been detected in the presynaptic terminals of neurons in DLB brain tissues with significant loss of postsynaptic dendritic spines, suggesting the role of such granular α-Syn aggregates in synaptic dysfunction (Kramer and Schulz-Schaeffer 2007). Tauopathies, even though are pathologically characterized by aggregates of tau protein, exhibit unique tau aggregates, which is different in each disease (Ballatore, Lee et al. 2007, Gendron and Petrucelli 2009). This highlights to a more complicated scenario where each disease can have unique type of protein aggregate. In our previous section of study with in vitro α-Syn oligomeric strains, we observed that DA and DHA, the two physiologically relevant inducers can modify recombinant monomeric α-Syn into totally two different oligomers that varied in their biological properties. Accumulating evident suggests that α-Syn can undergo different conformational states, resulting in different aggregates. Despite increasing amount of work being performed in this area, the mechanism by which α-Syn induces its toxicity is still elusive.

Biochemical and biophysical characterization of BDSOs showed they have similar sizes and morphologies as studied by Western blot and AFM analyses. All the BDSOs showed increased fluorescence binding with bis-ANS, suggesting that they are comparable to the in vitro SynO-aCSF. Although the BDSOs did not show any visible differences in

their biochemical and biophysical properties, digestion with PK enzyme revealed differences in their sensitivity to proteolysis. The three BDSOs, SynO-AD, SynO-DLB and SynO-PD showed different patterns of fragmentation upon PK digestion, suggesting them as strains of BDSOs. Incubation of primary cortical neurons from Tg-hSyn mice with the three BDSOs showed different patterns of accumulation of these oligomers. SynO-AD were mostly accumulated in the nuclear region, while SynO-DLB and SynO-PD were observed around and in the cell soma as well as in the processes. Future studies aiming at determining if this difference is disease specific or not will provide information on the cellular fate of these oligomers.

We observed that upon exogenously application of these BDSOs to the primary cortical neurons, their accumulation pattern inside the cells is different. It has been shown that the propagation of pathology relies on cell-to-cell spreading of pathogenic protein aggregates (Lee, Desplats et al. 2010, Reyes, Sackmann et al. 2019). Heparan sulfate proteoglycans (HSPGs), a family of proteins containing one or more sulfated glycosaminoglycan (GAG) heparan sulfate (HS) have been shown to be involved in cellular uptake of aggregated proteins, such as fibrils of α-Syn, amyloid-β (Kanekiyo, Zhang et al. 2011) and tau (Holmes, DeVos et al. 2013). A more recent study has shown that exosome-associated oligomeric α-Syn can be internalized in cells via HSPGs (Holmes, Furman et al. 2014, Delenclos, Trendafilova et al. 2017). Studies have shown that the relationship between GAGs and amyloidogenic proteins is complex in nature, which have been described in neurodegenerative diseases (Papy-Garcia, Christophe et al. 2011). Earlier, we have observed that in addition to dynamin-mediated endocytosis, both DA- and DHA-induced α-Syn oligomers can also be internalized into the cells via HSPG-mediated

endocytic pathway (Chapter 2). In this study we investigated if HSPGs are involved in the uptake of disease-relevant α-Syn oligomers (BDSOs) in the primary neurons. All the oligomers were shown to colocalize with HSPG endocytic receptor around the cell soma and the processes, but to different degrees. The most profound colocalization was noticed for SynO-DLB and SynO-PD with correlation coefficient of 0.86 and 0.81, respectively. This indicates a strong binding between these two oligomers and HSPG molecules. On the other hand, SynO-AD comparatively a less colocalization with HSPG, even though a considerable amount of these oligomers were internalized into the cells. This suggests that other endocytic pathways might be at play for the uptake of SynO-AD, while HSPG-mediated endocytosis may play an important role in the uptake of SynO-DLB and SynO-PD. Further investigation will enable us to elucidate the intracellular fate of these different BDSOs after being internalized.

Connexins are the main proteins in vertebrate gap junctions with almost 21 different connexin proteins. It is known that gap junctions are critical in maintaining cell-to-cell communication in the nervous system. They also maintain brain homeostasis between neuronal and non-neuronal cells (Collignon, Wetjen et al. 2006). As Cx43 is abundantly expressed in the brain cells, most of the work has been done on Cx43 protein (Vicario, Zappala et al. 2017). However, there is limited published information on Cx50 in the brain cells and moreover, its association with neurodegenerative diseases. Remarkably, here we have demonstrated that Cx50 binds to the BDSOs at different degrees, suggesting its involvement in the uptake of oligomers in the cells. Taken together, our results show that BDSOs possess considerable heterogeneity in their functional properties and also suggest a novel mechanism that is selective for uptake of different BDSOs. Here, we have

demonstrated the occurrence of Cx50 in human brain tissues, which has not been investigated before. We have also shown the co-occurrence of Cx50 and α-Syn protein in human brain tissues, specifically in Synucleinopathies. Further studies are needed to elucidate the cell-type specific expression of Cx50 in human brain tissues and the role of its association with α-Syn protein in disease pathology.

CONCLUSION

We observed that disease associated BDSOs were diverse in their functional properties. Upon exogenously application of these BDSOs to the primary cortical neurons, they accumulated differently inside the cells. The BDSOs also bound to heparan sulfate on the cell soma surface and/or in the processes to different degrees. More notably, here we have demonstrated a novel mechanism that might be involved in the uptake of oligomers in the cells. To the best of our knowledge, this is the first study showing functional heterogeneity among disease relevant α-Syn oligomers and the association of Cx50 with α-Syn oligomers in the neuronal cells, suggesting its potential involvement in the uptake of pathogenic α-Syn oligomers.

Parts of the Methods section have been taken from "Sengupta U, Montalbano M, McLean S, Kharas M, Kayed R (2018). Formation of Toxic Oligomeric Assemblies of RNA-binding Protein: Musashi in Alzheimer's disease. Acta Neurophathol Commun 6:113. doi: 10.1186/s40478-018-0615-0."

CHAPTER 4

STRAIN-SPECIFIC INTERACTION OF ALPHA-SYNUCLEIN OLIGOMERS WITH TAU AND ITS EFFECTS IN TAU AGGREGATION

ABSTRACT

Although α-Syn aggregation is primarily viewed in Synucleinopathies, co-occurrence of both α-Syn and tau have long been studied in these diseases. Here, we have studied the cross-seeding effect of the two recombinant α-Syn oligomer strains induced by dopamine (DA) or docosahexaenoic acid (DHA) as well as three polymorphic α-Syn oligomer preparations derived from Alzheimer's disease (AD), Parkinson's disease (PD) and dementia with Lewy bodies (DLB) brain tissues (BDSOs) on tau aggregation. Using molecular and cellular techniques, we observed that the tau aggregates cross-seeded with these specific α-Syn oligomeric strains were also different in their biochemical and biological properties, thus suggesting distinct aggregated tau strains. The DA-modified α-Syn oligomeric strain cross-seeded a tau aggregate strain that possessed a more potent seeding propensity than the tau aggregate prepared by cross-seeding with the DHA-modified α-Syn oligomeric strain. Among the BDSOs cross-seeded tau aggregates, PD and DLB BDSOs cross-seeded tau aggregates showed significantly increased propensities to seed for intracellular tau aggregation. However, the BDSOs cross-seeded tau aggregates were almost three times more potent seeds than the tau aggregates cross-seeded with

recombinant α-Syn oligomers. This study provides a comprehensive analysis of unique strain-specific interaction between oligomeric α-Syn and tau, allowing us to speculate that distinct α-Syn-tau interactions inducing tau aggregation can be an underlying mechanism of neurodegeneration in Synucleinopathies.

BACKGROUND

Many neurodegenerative diseases share common features in terms of their late-life clinical manifestations as well as protein aggregation, loss of synapse and cell death as revealed by the post-mortem histopathological analyses (Gadad, Britton et al. 2011). Neurodegenerative diseases-associated pathogenic proteins often interact with one another and modulate downstream activities that disrupt their normal functions (Clinton, Blurton-Jones et al. 2010). Even though the major pathological hallmarks of synucleinopathies are α-Syn aggregates, pathology of abnormal tau aggregates, NFTs, have long been studied in DLB, PD and MSA diseases (Jellinger 2009, Moussaud, Jones et al. 2014). As mentioned earlier, DLB and AD share many clinical and histopathological features, making the diagnosis of DLB a challenge. Co-morbidity of AD and DLB is a major and common problem, where almost 85% of DLB cases are shown with abundant amyloid plaques, a pathological hallmark of AD (Gomperts, Locascio et al. 2016). Moreover, LBs found in PD and DLB cases also contain hyperphosphorylated tau (Arima, Hirai et al. 1999, Iseki, Takayama et al. 2002). Similarly, aggregated α-Syn pathology is also demonstrated in AD, a major tauopathy (Larson, Sherman et al. 2012). At autopsy, more than half of AD patients showed LBs(Hamilton 2000). Several neurodegenerative disorders that are pathologically characterized by α-Syn protein aggregation, are also associated with aggregates of the microtubule-associated protein tau into NFTs such as PD (Spillantini, Schmidt et al. 1997,

Ishizawa, Mattila et al. 2003) , PDD, DLB and MSA (Iseki, Takayama et al. 2002, Lei, Ayton et al. 2010, Nagaishi, Yokoo et al. 2011, Colom-Cadena, Gelpi et al. 2013). Recent analyses of animal models of these diseases suggest that true neurodegeneration phenotypes correlate with the accumulation of soluble tau oligomers but not NFTs. Exogenous fibrils of recombinant tau or α-Syn can be taken up in cells and seed the fibrillization of tau and α-Syn respectively (Badiola, de Oliveira et al. 2011). Transgenic mouse model overexpressing human α-Syn mutation, A53T, showed abundant tau pathology in several brain regions (Giasson, Forman et al. 2003), whereas in another study, transgenic mouse model overexpressing human wild type (WT) α-Syn, exhibited spontaneous age-dependent tau pathology (Haggerty, Credle et al. 2011).

Our laboratory was the first to demonstrate that, in addition to α-Syn oligomers, tau oligomers were also present in the brain tissues of PD and DLB patients(Sengupta, Guerrero-Munoz et al. 2015). We have previously reported that α-Syn oligomers are capable of forming tau oligomers (Lasagna-Reeves, Castillo-Carranza et al. 2010). In our recent study, we have demonstrated that α-Syn oligomers induce a distinct and more potent tau oligomeric strain. Oligomeric complexes of α-Syn and tau derived from the brain tissues of PD patient, accelerated endogenous tau oligomer formation, caused memory deficits and spread disease pathologies as compared to the pure tau oligomers isolated from PSP (Castillo-Carranza, Guerrero-Munoz et al. 2018).

Here we have studied the cross-seeding effect of the polymorphic α-Syn oligomers inducing tau aggregation. We have used well-characterized recombinant α-Syn oligomeric strains as well as BDSOs isolated from AD, PD and DLB brain tissues. The cross-seeded tau aggregates were thoroughly characterized for their biochemical, biophysical and

biological properties. To the best of our knowledge this is the first study showing unique α-Syn oligomeric strain-specific interaction with tau, allowing us to speculate that distinct α-Syn-tau interactions inducing tau aggregation can be an underlying mechanism of neurodegeneration in Synucleinopathies.

METHODS

Tau Aggregation Assay

Human recombinant full-length wild-type (Wt) 2N4R tau (tau 441) was expressed and purified to obtain monomeric tau. To prepare BDSOs cross-seeded tau aggregates, seeds of α-Syn oligomer derived from each pathology were added to 1 mL tau monomer (0.5 μg/μl), at 1:140 ratio (Lasagna-Reeves, Castillo-Carranza et al. 2010) and incubated for 24 h on an orbital shaker at RT.

Western blot Analysis

Approximately 1-2 sample were. For electrophoresis with tau aggregates, approximately 2 μg of each tau aggregate sample was loaded on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked with 10% nonfat dry milk at 4°C overnight. Membranes were then probed with primary antibodies, T22 (1:250) and Tau 5 (1:5000; Bio Legend, 806402) diluted in 5% nonfat dry milk for 1 h at RT. HRP-conjugated anti-mouse IgG (1:6000, GE Healthcare) was used to detect Tau 5 immunoreactivity, whereas an HRP-conjugated anti-rabbit IgG (1:6000, GE Healthcare) was used for T22 immunoreactivity. ECL plus (GE Healthcare) was used to visualize the bands.

Densitometric analysis was performed using ImageJ software (National Institutes of Health).

Atomic Force Microscopy

Approximately 3-4 µl of each aggregated tau sample was applied onto a fresh-cleaved mica surface and imaged using a non-contact tapping method with a Multimode 8 AFM machine (Bruker, Billerica MA) as described in Chapter 2.

Bis-ANS and Thioflavin T Fluorescence Assays

Three µl of tau aggregates (0.6 µg/ µl) and 247 µl of 10 µM bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt, Invitrogen) prepared in mM glycine-NaOH buffer (pH 7.4) were added to the wells of 96-well clear-bottomed black plates. Each condition was performed in triplicates. The fluorescence intensity was measured at λ-emission 520 nm upon λ-excitation 380 nm. For Thioflavin T (ThT) assay, 3 µl of protein (0.6 µg/ µl) and 247 µl of 20 µM ThT prepared in 50 mM glycine-NaOH buffer (pH 8.5) were added in triplicates to the wells. Fluorescence intensity was read at λ-emission 490 nm following excitation at 440 nm using a POLARstar OMEGA plate reader (BMG Lab technologies). Each condition for this assay was performed in triplicates.

Proteolytic Digestion of Aggregated Tau by Proteinase K Enzyme

Aggregated tau samples (~ 3 µg) were treated with proteinase K enzyme at 0-1 µg/mL in presence of Tris-HCl and NaCl (100 mM and 5 mM final concentrations, respectively) and incubated at 37^0C for 1 h. At the end of incubation time, 1 X LDS sample buffer (Invitrogen) was added and heated at 95 ^{0}C for 10 min. Samples were immediately transferred onto ice to stop the cleavage reaction followed by loading the digestion products

into 4-12% bis-tris precast gels (Invitrogen) for SDS-PAGE gel electrophoresis. Western blot analysis with generic tau antibody, Tau 5, was performed to visualize the digested fragments.

Tau RD P301S Biosensor cell culture and seeding assay

Tau RD P301S biosensor cells (ATCC; CRL-3275) were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were maintained in a humidified atmosphere equipped with 5% CO_2 at 37°C. For seeding assay, cells were plated at a density of 1 x 10^5 cells/well in 24-well plates on poly-L-Lysine-coated coverslips. After 18 h, cells were transduced with three preparation of aggregated tau with Lipofectamine 2000 (Invitrogen) mixed in Opti-Mem (Gibco) medium following a protocol published elsewhere (Holmes, Furman et al. 2014) . Aggregated tau and Liposome mixtures were incubated at RT for 30 min before adding to the tau biosensor cells. Cells were incubated for 24 h followed by washing 3 times with PBS. Coverslips were fixed with 4% formaldehyde and mounted with Prolong Gold mounting media for imaging.

Statistical Analysis

All statistical analyses were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). All values were calculated as mean and standard deviation (SD). Data are presented from at least 3 replicates and from 3 independent experiments. For FRET positive cells quantification, two-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed. For fluorescent correlation measurement and bis-ANS

and thioflavin T fluorescence, one-way ANOVA with Tukey's multiple comparisons test was performed. The number of experiments is mentioned in the figure legends.

RESULTS

DA- and DHA-induced α-Syn Oligomeric Strains Cross-seed into Different Aggregated Tau Strains

Previously we have demonstrated that α-Syn oligomers act as seeds initiating tau aggregation by forming tau oligomers. Such α-Syn oligomer cross-seeded tau oligomers appeared to be toxic on different cell lines, including primary cortical neurons (Castillo-Carranza, Guerrero-Munoz et al. 2018). Therefore, next we sought to investigate what are the roles of the two α-Syn oligomeric strains in tau aggregation process. We used strains of α-Syn oligomers as seeds to initiate aggregation of monomeric tau which are referred to

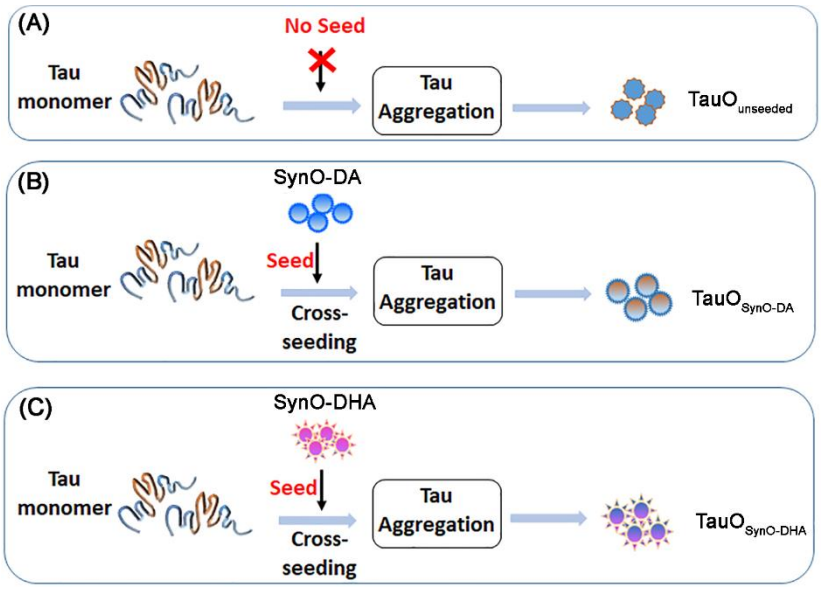

Figure 4. 1 Schematic representation of tau aggregates cross-seeded with recombinant α-Syn oligomeric strains.
Tau aggregates formed without using any α-Syn oligomer as seed (**A**), as well as cross-seeded with α-Syn oligomeric strains, SynO-DA (**B**) and SynO-DHA (**C**). The tau aggregates are referred to as $TauO_{unseeded}$, $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$, respectively.

as $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ based on their respective seeds as schematically illustrated in **Figure 4.1A-C**. As a control, we also aggregated monomeric tau without any α-Syn oligomer seed which is mentioned here as $TauO_{unseeded}$ (**Figure 4.1A**). We thoroughly characterized such tau aggregates formed by cross-seeding with α-Syn strains as well as unseeded by biochemical, biophysical and biological methods.

Representative WB image of three tau aggregates immunolabeled with T22 antibody showed higher aggregates of ~75kDa and above molecular weights (**Figure 4.2A**). However, $TauO_{SynO\text{-}DA}$ showed stronger signal appearing higher than ~150 kDa in $TauO_{SynO\text{-}DHA}$ or $TauO_{unseeded}$. Upon probing with Tau 5, a generic sequence specific anti-tau antibody, both $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ showed strong signal around ~150 kDa and higher molecular weight compared to $TauO_{unseeded}$. Nevertheless, Tau 5 antibody signal was consistent with T22 for $TauO_{SynO\text{-}DA}$ which was higher than $TauO_{SynO\text{-}DHA}$ (**Figure 4.2A**). To evaluate if there were any morphological differences between the two cross-seeded tau aggregates, we performed AFM analysis. Representative AFM images showed that $TauO_{SynO\text{-}DA}$ contained aggregates larger than $TauO_{SynO\text{-}DHA}$ (**Figure 4.2B**). These aggregates were also analyzed by SEC (**Figure 4.2C-E**). Although, the patterns of peaks corresponding to the aggregates appeared to be similar for the two cross-seeded tau aggregates, but the elution time was different. Next, we performed bis-ANS and ThT fluorescence assays to measure the hydrophobicity of the tau aggregates. Binding of bis-ANS to the three tau aggregates was significantly higher compared to fibrils (**Figure 4.2F**). Notably, $TauO_{SynO\text{-}DA}$ showed stronger binding affinity than $TauO_{SynO\text{-}DHA}$, while, ThT bound strongly to the fibrils compared to the three oligomeric aggregates (**Figure 4.2G)**. Finally, to evaluate if the tau aggregates formed by the cross-seeding of the two α-Syn

strains, are tau strains or not, we performed PK digestion following our published method

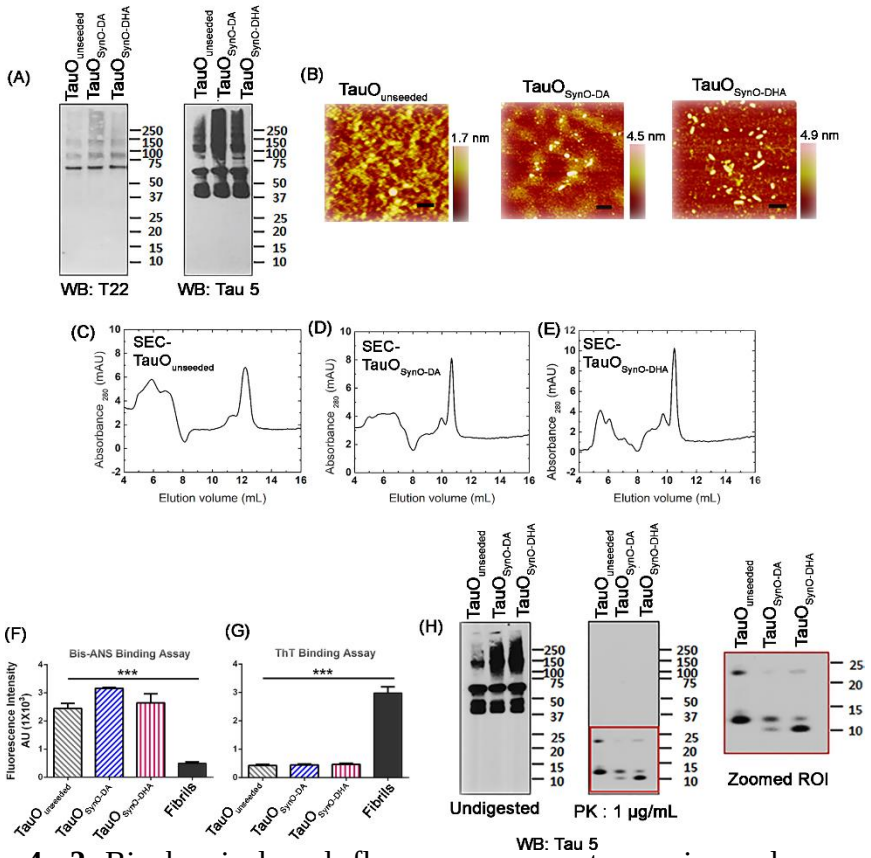

Figure 4. 2 Biochemical and fluorescence spectroscopic analyses of tau aggregates.

(**A**) Representative WB image with tau oligomer specific antibody, T22, showing higher molecular weight aggregates. Sequence-specific tau antibody, Tau 5 detects different forms of tau aggregates in these samples. (**B**) AFM images of unseeded and cross-seeded tau aggregates. (**C-E**) Size exclusion chromatograms of the three tau aggregates (**F**) Fluorescence intensity of bis-ANS binding to all tau aggregates. Bis-ANS binding to all 3 preparations of tau aggregates is significantly strong compared to fibrils. (**G**) Fluorescence intensity of ThT binding to tau aggregates. Unseeded and the 2 cross-seeded tau aggregates show less binding affinity for ThT compared to fibrils. (**H**) PK digestion profile for the tau aggregates at 1 µg/mL PK concentration showing different cleavage patterns. Zoomed region of interest (ROI) shows fragments generated from three tau aggregate samples after PK digestion. Data are represented as mean ± SD from 4 replicates performed in 3 independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparisons test. *** $p < 0.001$. Scale bar 100 nm.

(Sengupta, Carretero-Murillo et al. 2018). $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ were digested with PK enzyme at 1 µg/mL concentration followed by WB analysis with generic anti-tau

antibody, Tau 5 (**Figure 4.2H**). Intriguingly, the two cross-seeded tau aggregates showed completely different patterns of fragments upon PK digestion. Fragments generated by $TauO_{SynO\text{-}DA}$ were smaller than fragments of $TauO_{SynO\text{-}DHA}$, indicating the difference in their protease-sensitive cores. The cross-seeded tau aggregates varied in their stability and conformation, suggesting them as two different strains of tau aggregates.

Polymorphic BDSOs Cross-seeded Tau Aggregates Show Polymorphism

To evaluate the effects of BDSOs on tau aggregation, we prepared tau aggregates by using BDSOs and SynO-aCSF as seeds (**Figure 4.3**). Each cross-seeded tau aggregate

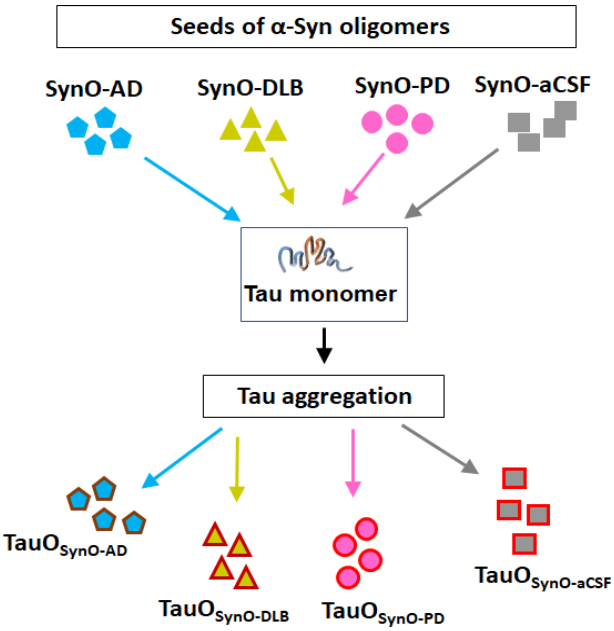

Figure 4. 3 Schematic representation of tau aggregates cross-seeded with BDSOs.
The three BDSOs: SynO-AD, SynO-DLB and SynO-PD, as well as SynO-aCSF were used as seeds to aggregate tau as depicted by different shapes and colors. The tau aggregates are referred to as $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$, $TauO_{SynO\text{-}PD}$ and $TauO_{SynO\text{-}aCSF}$, respectively.

is labeled with their corresponding seed name. Next, we evaluated the biochemical and biophysical properties of the tau aggregates formed by cross-seeding with the three BDSOs, such as SynO-AD, SynO-DLB and SynO-PD as well as with SynO-aCSF.

Henceforth, the four cross-seeded tau aggregates are termed as $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$, $TauO_{SynO\text{-}PD}$ and $TauO_{SynO\text{-}aCSF}$, respectively. Representative WB image of the four tau aggregates immunolabeled with T22 antibody showed aggregates of ~75kDa and above

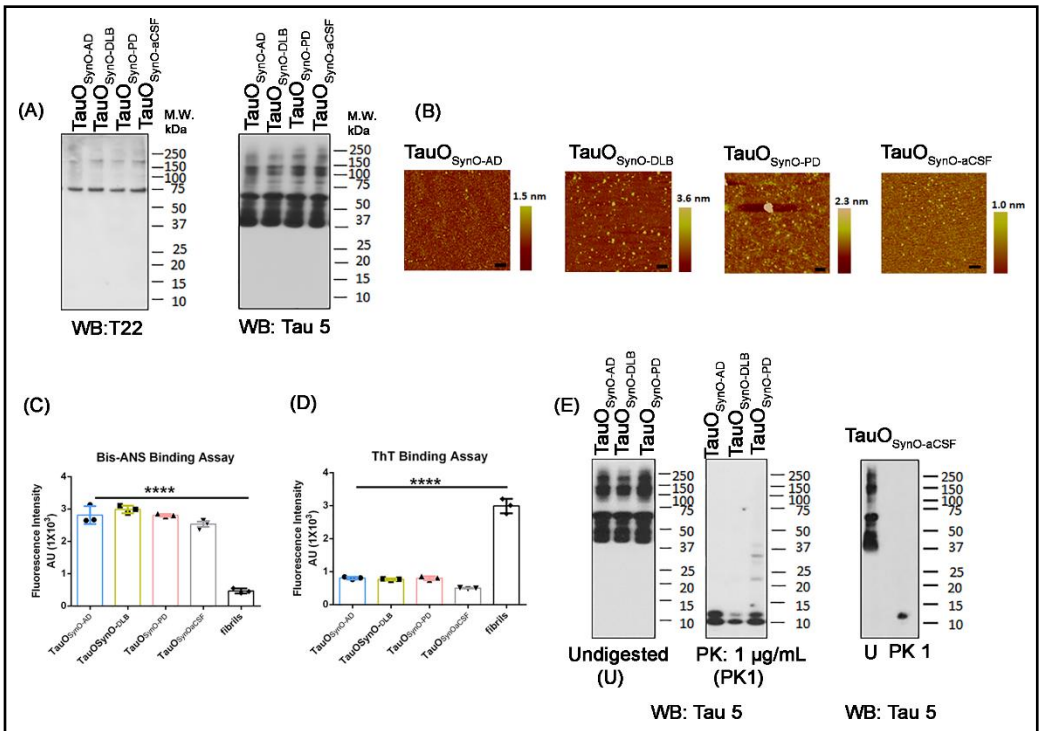

Figure 4. 4 Biochemical and fluorescence spectroscopic analyses of tau aggregates cross-seeded with BDSOs and SynO-aCSF.
(**A**) Representative WB image with tau oligomer specific antibody, T22, showing higher molecular weight aggregates. Sequence-specific tau antibody, Tau 5 detects different forms of tau aggregates in these samples. (**B**) AFM images of BDSOs and SynO-aCSF cross-seeded tau aggregates. (**C**) Fluorescence intensity of bis-ANS binding to all tau aggregates. Bis-ANS binding to all four preparations of tau aggregates was significantly strong compared to fibrils. (**D**) Fluorescence intensity of ThT binding to tau aggregates. The cross-seeded tau aggregates showed less binding affinity for ThT compared to fibrils. (**E**) PK digestion profile for the tau aggregates at 1 µg/mL PK concentration showing different cleavage patterns. Data are represented as mean and SEM from 3 replicates performed in 3 independent experiments. One-way ANOVA with Tukey's multiple comparisons test. **** $p < 0.0001$. Scale bar 100 nm.

molecular weights (**Figure 4.4A)**. Upon probing with Tau 5, a generic sequence specific anti-tau antibody, all the three BDSOs cross-seeded tau aggregates showed strong signal around ~150 kDa and higher molecular weight. To study the morphologies of the tau

aggregates, we performed AFM analysis. Representative AFM images showed that although they had spherical oligomeric structures, however, they had differences in the heights. $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ contained aggregates that were higher in heights than $TauO_{SynO\text{-}AD}$ or $TauO_{SynO\text{-}aCSF}$ (**Figure 4.4B**). We also performed bis-ANS and ThT fluorescence assays to measure the hydrophobicity of the tau aggregates. Binding of bis-ANS to the three tau aggregates was significantly higher compared to fibrils (**Figure 4.4C**), while, ThT bound strongly to the fibrils compared to the three oligomeric aggregates (**Figure 4.4D**).

Next we evaluated proteolytic stability of the tau aggregates formed by the cross-seeding with the three polymorphic BDSOs by subjecting these oligomers to PK enzyme digestion following our published method (Sengupta, Carretero-Murillo et al. 2018). $TauO_{SynO\text{-}AD,}$ $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ were digested with PK enzyme at 1 µg/mL concentration followed by WB analysis with generic anti-tau antibody, Tau 5 (**Figure 4.4E**). Intriguingly, the three cross-seeded tau aggregates showed different patterns of fragments upon PK digestion. Two small bands were observed in case of $TauO_{SynO\text{-}AD}$ and $TauO_{SynO\text{-}DLB}$, while $TauO_{SynO\text{-}PD}$ showed fewer higher bands, indicating comparatively a more resistant oligomer to proteolysis. This indicates the difference in the protease-sensitive cores of the different BDSOs. The cross-seeded tau aggregates varied in their stability, suggesting them as three different strains of tau aggregates.

Aggregated Tau Strains Cross-seeded with Recombinant α-Syn oligomeric strains Exhibit Distinct Tau Seeding

Previously it has been shown that tau aggregates present in human and mice brain lysates contained the effective seed causing tau aggregation in Tau-RD P301S-CFP/YFP biosensor cells (Holmes, Furman et al. 2014, Furman, Holmes et al. 2015).

Next, we sought to investigate if the two aggregated tau strains that were cross seeded with in vitro α-Syn oligomeric strains, SynO-DA and SynO-DHA, can act as seeds or not. The effective dose for seeding activity of the aggregated tau strains ($TauO_{SynO\text{-}DOPA}$ and $TauO_{SynO\text{-}DHA}$) was empirically determined by generating dose-response curves

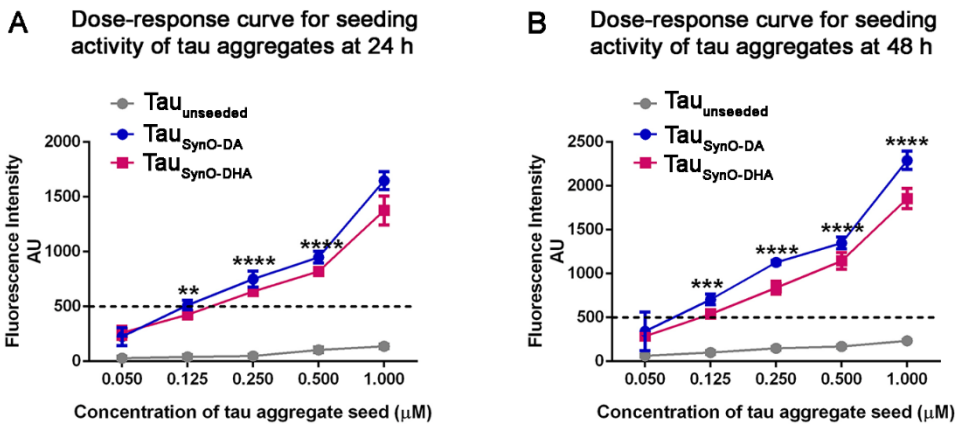

Figure 4. 5 Dose-response curves for seeding activity of tau aggregates. Tau biosensor cells were exposed to increased concentrations of the three tau aggregates (0.05, 0.125, 0.25, 0.5 and 1 µM) in presence of Lipofectamine and fluorescence intensity was measured at 24 h (**a**) and 48 h (**b**) time points. Data are represented as mean ± SD from four experimental replicates. Statistical significance was calculated using two-way ANOVA with Bonferroni post hoc analysis. ** p<0.01, *** p< 0.001, **** p<0.0001.

(**Figure 4.5**). Tau biosensor cells were exposed to increased concentrations of the three tau aggregates (0.05, 0.125, 0.25, 0.5 and 1 µM) in presence of Lipofectamine and fluorescence intensity was measured at 24h and 48 h time points. For both the cross-seeded tau aggregates, fluorescence intensity was detected at 0.25, 0.5 and 1 µM concentrations at 24 h (**Figure 4.5A**) and 48 h (**Figure 4.5B**), indicative of the tau inclusion formation. It is noteworthy to mention that at 48 h time point, even 0.125 µM concentration of seed was

able to produce significantly increased fluorescence intensity compared to 0.05 µM concentration of seed. On the contrary, seed of $TauO_{unseeded}$ did not produce any detectable

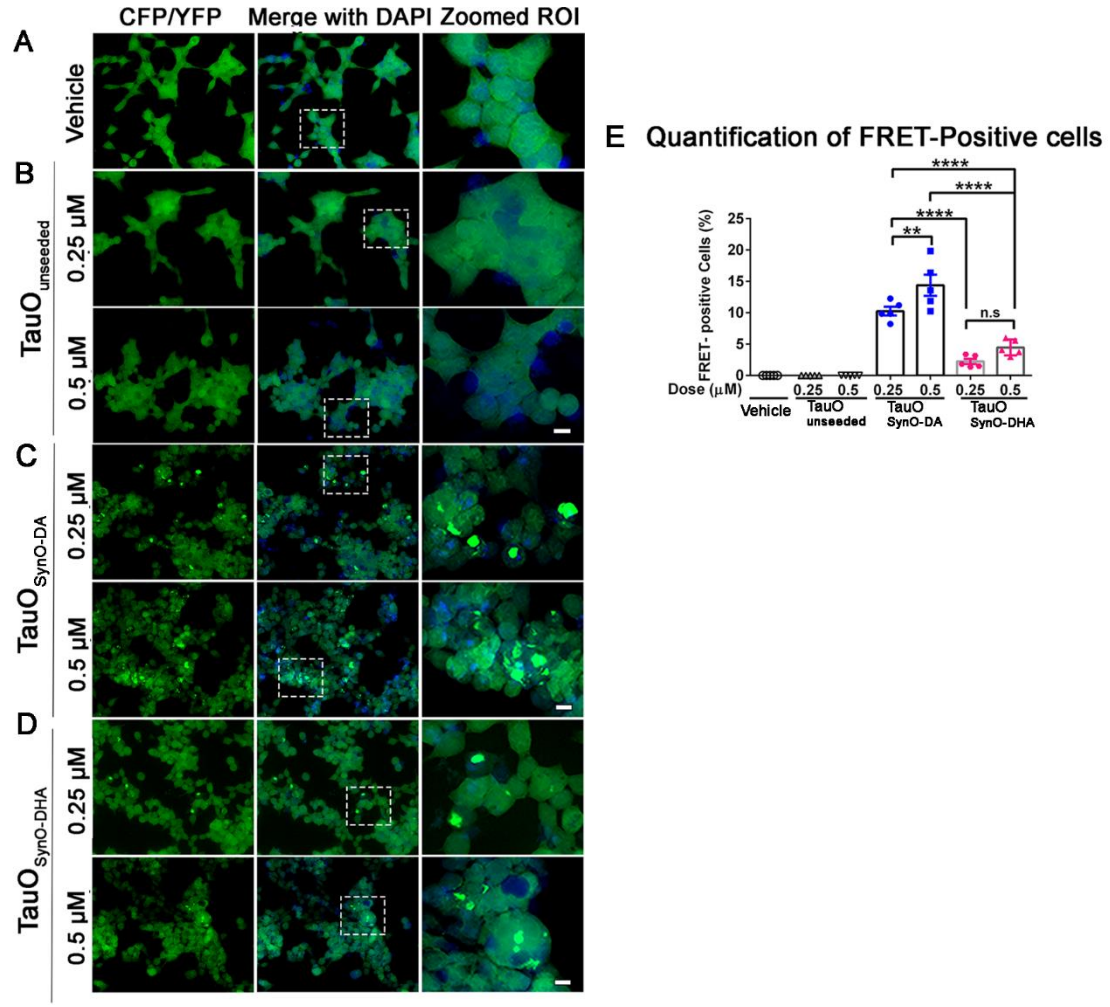

Figure 4. 6 Seeding assay of recombinant α-Syn oligomeric strains cross-seeded tau aggregate strains in Tau RD-P301S CFP/YFP FRET biosensor cells.
(**A-D**) Tau biosensor cells were exposed to the cross-seeded tau aggregates as well as unseeded tau aggregate at 0.25 and 0.5 µM concentrations for 24 h with lipofectamine. The cross-seeded tau aggregates, $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ show dose-dependent increased seeding propensity at the concentrations used resulting in the formation of cytosolic tau aggregates. Unseeded tau aggregates did not show any seeding. $TauO_{SynO\text{-}DA}$ has more potent seed efficiency than $TauO_{SynO\text{-}DHA}$. (**E**) Quantification of FRET positive cells observed in all 4 groups of treatment: vehicle, $TauO_{unseeded}$, $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$. Quantification is performed from 15 regions of interest (ROIs) from 3 replicates performed in 3 independent experiments. Statistical significance is measured by using two-way ANOVA with Bonferroni post hoc analysis. ** $p < 0.01$, **** $p < 0.0001$. Scale bar 10 µm

fluorescence at either 24 or 48 h. Based on the dose-response curves, we exogenously

added $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ to the tau biosensor cells grown on cover slips at 0.25 and 0.5 µM concentrations (**Figure 4.6A-D**). Simultaneously, we also used unseeded tau oligomers at the similar concentrations. We observed that the 2 tau aggregates formed by cross-seeding with two α-Syn strains acted as seeds forming tau inclusions in the biosensor cells at both concentrations. Since apparently, there was no tau aggregates formed in vehicle and $TauO_{unseeded}$ cells, we compared the seeding between $TauO_{SynO\text{-}DA}$ and $TauO_{SynO\text{-}DHA}$ treated groups. Although at lower concentration of 0.25 µM, these tau aggregates were able to seed, but at 0.5 µM concentration, the seeding was increased (**Figure 4.6E**). More notably, $TauO_{SynO\text{-}DA}$ was more effective seed than $TauO_{SynO\text{-}DHA}$. Even so, $TauO_{SynO\text{-}DA}$ showed significantly increased seeding capacity at 0.25 µM concentration than $TauO_{SynO\text{-}DHA}$ at 0.5 µM concentration. As mentioned above, $TauO_{unseeded}$ did not cause any visible seeding. These results suggest that the aggregated tau strains possess different seeding capacities.

BDSOs Cross-seeded Aggregated Tau Strains Exhibit Distinct Tau Seeding

Although the three different BDSOs can cross-seed into polymorphic aggregated tau strains, they might not be functionally relevant at the same degree. Next, to evaluate the seeding properties of the BDSOs cross-seeded tau aggregates, we exogenously added $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ to the tau biosensor cells at 0.25 and 0.5 µM concentrations (**Figure 4.7B-D)**. Simultaneously, we also used $TauO_{SynO\text{-}aCSF}$ at the similar concentrations and vehicle for comparison (**Figure 4.7A, E)**. The disease associated aggregated tau strains: $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ showed significantly increased seeding propensity with increasing concentration of seeds (0.25 µM vs 0.5 µM). $TauO_{SynO\text{-}AD}$ showed comparatively less seeding potency of intracellular tau inclusion

formation than $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ at 0.25 µM concentration (**Figure 4.7B-D**).

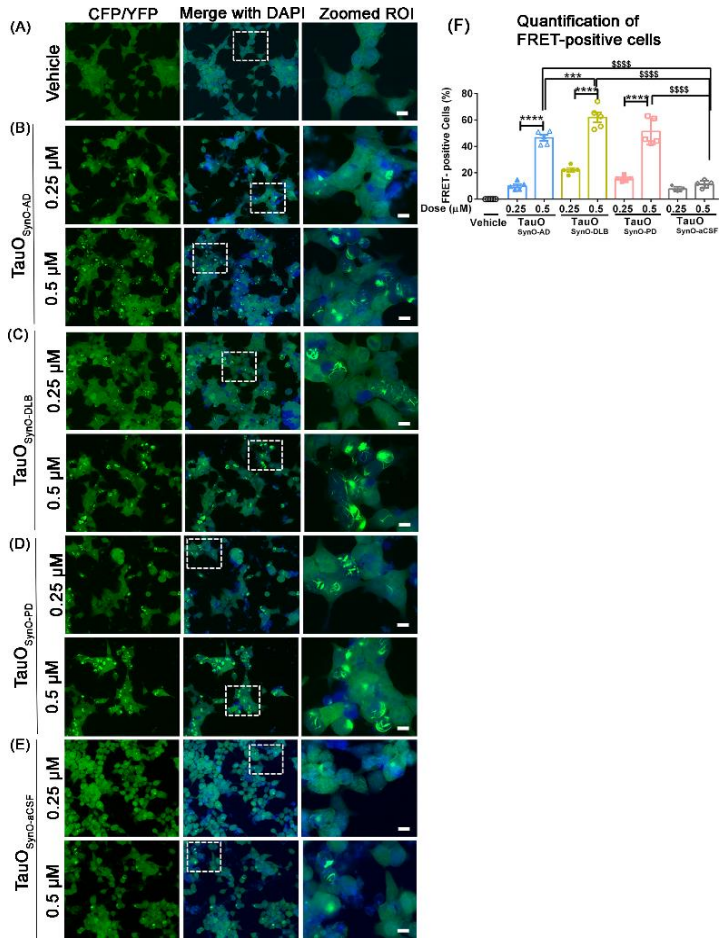

Figure 4. 7 Seeding assay of BDSOs cross-seeded tau aggregate strains in Tau RD-P301S CFP/YFP FRET biosensor cells.
(A-E) Tau biosensor cells were exposed to the BDSOs and SynO-aCSF cross-seeded tau aggregates at 0.25 and 0.5 µM concentrations for 24 h with lipofectamine. The cross-seeded tau aggregates, $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$, $TauO_{SynO\text{-}PD}$ and $TauO_{SynO\text{-}aCSF}$ showed dose-dependent increased seeding propensities at the concentrations used resulting in the formation of cytosolic tau aggregates. Compared to the BDSOs cross-seeded tau aggregates, SynO-aCSF cross-seeded tau aggregate showed significantly less potency for seeding. As expected, vehicle treatment did not show any signal. **(F)** Quantification of FRET positive cells observed in all 5 groups of treatment: vehicle, $TauO_{SynO\text{-}AD}$, $TauO_{SynO\text{-}DLB}$ and $TauO_{SynO\text{-}PD}$ showed significantly increased seeding propensity as compared to $TauO_{SynO\text{-}aCSF}$ 0.5 µM concentration. Quantification was performed from fifteen regions of interest (ROIs) from three replicates performed in three independent experiments. Statistical significance is measured by using two-way ANOVA with Bonferroni post hoc analysis. *** $p < 0.001$, **** $p < 0.0001$, $^{\$\$\$\$}$ $p < 0.0001$. Scale bar 10 µm.

At 0.5 μM concentration, all the three BDSOs cross-seeded aggregated tau showed significantly increased amount of tau inclusion formation. TauO $_{\text{SynO-aCSF}}$ seeded a negligible amount of inclusion. More notably, BDSOs cross-seeded aggregated tau strains were much more potent seeds for tau inclusion formation than recombinant SynO-aCSF cross-seeded tau aggregate at both 0.25 and 0.5 μM concentrations. Vehicle control did not show any tau inclusion formation (**Figure 4.7A, E**).

DISCUSSION

Pathogenic protein aggregates can act as seeds inducing toxic accumulation of the same protein or other aggregation-prone proteins, thus representing an overlap between many protein pathologies. Our laboratory and other groups have demonstrated that α-Syn cross-seeds tau protein, promoting its aggregation (Giasson, Forman et al. 2003, Lasagna-Reeves, Castillo-Carranza et al. 2010), suggesting a direct interaction. The co-occurrence of α-Syn and tau in multiple diseases suggests their orchestrated method of toxicity (Moussaud, Jones et al. 2014). In yeast, co-expression of both α-Syn and tau led to more deleterious effects than α-Syn or tau protein alone(Ciaccioli, Martins et al. 2013). A recent study from our laboratory has demonstrated that α-Syn-induced tau aggregate is driven towards a more toxic state compared to tau aggregate without α-Syn (Castillo-Carranza, Guerrero-Munoz et al. 2018).

In this study, we have explored the effect of α-Syn oligomeric strains on tau aggregation. Tau aggregates formed by cross-seeding with two recombinant α-Syn oligomeric strains (SynO-DA and SynO-DHA) exhibited differences in their biochemical and biophysical properties. Remarkably, the two cross-seeded tau aggregates showed different patterns of fragmentation upon digestion with PK, suggesting their variability in

conformation and stability. The Tau-RD P301S-CFP/YFP biosensor cell line developed by Diamond M et al., provides an useful tool to measure the seeding activity as a functional aspect of tau aggregates (Furman, Holmes et al. 2015, Chung, Carlomagno et al. 2019). In our study, we investigated the seeding propensity of the two aggregated tau strains by exogenously adding them in the tau biosensor cells. We anticipated that not every α-Syn oligomer strain would lead to a biologically relevant tau aggregate strain. Surprisingly, our findings here demonstrate that DA modified α-Syn oligomers cross-seeded tau is a more potent seed causing increased tau aggregation. Although, DA modified α-Syn oligomeric strain is less toxic in cultured cell lines than DHA modified α-Syn strain, tau aggregate cross-seeded with DA modified α-Syn oligomer strain is a biologically more effective seed.

In our recent study, we have demonstrated that in Htau mice, intracerebral injection of oligomeric complexes of α-Syn and tau from PD brain tissues caused more robust pathology than only tau oligomers isolated from brain tissues of PSP (Castillo-Carranza, Guerrero-Munoz et al. 2018). This suggested that the oligomeric α-Syn and tau interaction and therefore, their cross-seeding mechanism is crucial to disease pathology. Next, we evaluated if the BDSOs can cross-seed into functional tau aggregates. The tau aggregates cross-seeded with the three BDSOs appeared to be similar in Western blot analysis. However, they varied in their morphologies as observed by AFM imaging. Importantly, upon proteolysis digestion, the tau aggregates showed different patterns of fragmentation. While $TauO_{SynO\text{-}AD}$ and $TauO_{SynO\text{-}DLB}$ showed complete cleavage of the aggregates showing mainly two smaller bands, $TauO_{SynO\text{-}PD}$ retained some remnants of the higher aggregates. These results indicate that the three polymorphic tau aggregates can be regarded as three cross-seeded tau strains. All the three disease associated tau aggregates were capable of

significantly forming tau inclusion in the tau biosensor cells, thus indicating their potencies as seeds. Interestingly, tau aggregates cross-seeded with BDSOs were almost three times more potent seeds than the ones cross-seeded with recombinant α-Syn oligomeric strains. This suggests that protein seeding is a crucial mechanism in disease pathogenesis. Our observation here also suggests that the diversity noticed here between the aggregated tau strains might be due to the interaction between tau and different types of α-Syn oligomers or oligomeric strains that may occur in different diseases. We have reported for the first time the existence of biologically relevant α-Syn oligomeric strains cross-seeded tau aggregate strains that can be differentiated based on their stability to proteolysis and seeding propensity forming cellular tau aggregates.

CONCLUSION

Currently, there is an increasing effort to understand co-occurring protein pathologies that are frequently observed in neurodegenerative diseases and have complicated both pathophysiological investigations, as well as treatment development. Thus, our findings regarding the strain-specific interaction between α-Syn and tau open new avenues for neuroprotective intervention strategies for Synucleinopathies by specifically targeting these stable toxic oligomers.

Part of the Method section has been taken from "Sengupta U, Montalbano M, McLean S, Kharas M, Kayed R (2018). Formation of Toxic Oligomeric Assemblies of RNA-binding Protein: Musashi in Alzheimer's disease. Acta Neurophathol Commun 6:113. doi: 10.1186/s40478-018-0615-0."

CHAPTER 5

CONCLUSIONS AND SIGNIFICANCE

Neurodegenerative diseases are a group of devastating diseases that affect day to day life by impairing memory or movement, thus significantly deteriorating the quality of life (Przedborski, Vila et al. 2003). With increasing life expectancy, the incidences of these diseases are increasing, imposing a tremendous burden on socio-economic status world-wide (Masuda-Suzukake, Nonaka et al. 2013). Apart from the clinical symptoms, these degenerative diseases are pathologically characterized by the presence of proteinaceous aggregates. It is well established that protein misfolding and pathogenic aggregation leading to abnormal accumulation is the key event of neurodegeneration(Ross and Poirier 2005). However, there is a considerable overlap among the clinical symptoms and pathological protein aggregates between multiple diseases (Ahmed, Devenney et al. 2016). Aggregation of α-Syn in LBs and LNs, is a hallmark feature in Synucleinopathies. Since its discovery as the main component of these aggregates, there has been a continuous effort to better understand the underlying mechanism of α-Syn toxicity. Despite significant advances made in recent years, the exact reasons behind selective vulnerability of different neuronal and non-neuronal cell populations as well as brain regions to the α-Syn protein aggregation remain unknown. Implication of soluble α-Syn oligomers impairing multiple cellular processes has led to the consideration of such oligomeric assemblies as the main pathogenic entity in Synucleinopathies. Hence, finding answers for the key questions leading to α-Syn oligomers formation, seeding and propagation are of utmost importance. Collaborative studies involving multi-disciplinary approaches is required to better understanding of these debilitating diseases and identify disease-modifying intervention.

The co-occurrence of α-Syn and tau protein pathologies in several brain diseases implies a toxic relationship and has become an active area of research. Two of the major challenges faced in the neurodegeneration field are the formation and biological relevance of amyloid polymorphisms (strains) and the toxic interactions between amyloidogenic proteins (Gerson, Mudher et al. 2016). Previously, we demonstrated that, in addition to the meta-stable oligomeric α-Syn, tau oligomers are also present in Parkinson's disease and Dementia with Lewy bodies (Sengupta, Guerrero-Munoz et al. 2015).

Here, I have studied the effects of physiological inducers such as dopamine (DA) and docosahexaenoic acid (DHA) in modulating the aggregation, specifically the oligomerization of recombinant α-Syn protein (Chapter 2). The main findings from this study include:

- Oligomeric strains formed by these two physiological inducers possessed distinct structural properties in terms of their secondary structures and proteolytic stability to digestion by proteinase K enzyme. DA-induced α-Syn oligomers were more resistant to proteolysis than DHA-induced α-Syn oligomers. Furthermore, they showed different patterns of cleavage upon tryptic digestion
- The two oligomeric strains also showed diversity in their toxic properties studied in two different cell lines as well as their propensities to seed cytosolic α-Syn protein into aggregates
- Both the oligomeric strains were capable of significant reduction of dendritic spines at sub-lethal dose
- Inhibition of α-Syn oligomeric strains' internalization by a heparan sulfate proteoglycan (HSPG) antagonist (Heparin) and a dynamin antagonist (Dynasore)

significantly reduced oligomer-induced cytotoxicity. This suggests the involvement of HSPG- and dynamin-mediated endocytic pathways in the internalization of DA- and DHA-induced α-Syn oligomeric strains

Sengupta U, Puangmalai N, Bhatt N, Garcia S, Zhao Y, Kayed R (2020). Polymorphic α-Synuclein Strains Modified by Dopamine and Docosahexaenoic Acid Interact Differentially with Tau Protein. Molecular Neurobiology (accepted)

To investigate α-Syn oligomers occurring in different diseases, I have isolated, purified and characterized α-Syn oligomers from different Synucleinopathies, including AD, DLB and PD (Chapter 3). The main findings from this study include:

- The three brain-derived α-Syn oligomers (BDSOs) showed differences in their morphologies and stability to proteolytic cleavage by proteinase K enzyme. The different patterns of fragments generated by the proteolysis indicate the structural variability of the three BDSOs
- Upon exogenously added to the primary cortical neurons from transgenic animal expressing human α-Syn protein, the three BDSOs accumulated differently inside the cells.
- The BDSOs bound to the cell surface heparan sulfate at variable degrees, suggesting a selective involvement of heparan sulfate proteoglycan for the uptake of different disease-associated α-Syn oligomers
- The BDSOs also bound to the gap junction protein Connexin50 (Cx50) in the cell soma and processes at different degrees, suggesting its potential involvement in the uptake of these oligomers. This also highlights the presence of Cx50 in the primary neurons as well in human brain tissues, which has not been shown before

***Sengupta U** et al, Polymorphism and Functional Heterogeneity in Brain-derived Synuclein Oligomers. (In preparation)*

To investigate the strain-specific interaction of α-Syn oligomers and tau, I have studied the interaction of well-characterized recombinant and brain-derived α-Syn oligomers in tau aggregation (Chapter 4). I have also investigated consequential effects of such strain-specific interaction of oligomeric α-Syn strains with tau. The main findings resulted from this study include:

- Recombinant α-Syn oligomeric strains induced by DA and DHA, cross-seeded into discrete aggregated tau strains that differed in their morphologies and proteolytic stability
- Such cross-seeded aggregated tau strains possessed distinct effects on cellular tau seeding and aggregation in tau biosensor cells

Sengupta U, Puangmalai N, Bhatt N, Garcia S, Zhao Y, Kayed R (2020). Polymorphic α-Synuclein Strains Modified by Dopamine and Docosahexaenoic Acid Interact Differentially with Tau Protein. Molecular Neurobiology (accepted)

- Well-characterized polymorphic BDSOs cross-seeded into aggregated tau strains that were diverse in their morphologies and sensitivity to proteolysis.
- Finally, BDSOs cross-seeded tau aggregates showed significantly enhanced seeding propensities for cellular tau inclusion formation, which was almost three time increased than the recombinant α-Syn oligomers cross-seeded aggregated tau strains.

Currently, there is no cure for neurodegenerative diseases, including AD, PD and DLB. The available therapies are all symptomatic. Proper diagnosis of these diseases poses a big challenge even for accurate symptomatic treatment. There is an urgent need for the development of therapeutics to slow the progression of the diseases. With the observation that protein pathology spreads from neuron-to-neuron, immunotherapy has become an active area of interest for clearing up extracellular protein aggregates and, thus, halting intercellular transmission. It is now accepted that minimizing the secretion of toxic oligomers and/or targeting the extracellular oligomers of α-Syn by immunotherapy might provide usefulness to stop the spreading (Brundin, Dave et al. 2017). Association of α-Syn with Synucleinopathies, especially with PD pathogenesis has been established more than two decades ago. Although, the role of α-Syn protein in synaptic plasticity has been demonstrated, the mechanisms through which this presynaptic protein acquires its toxic function, is still under extensive investigation. The development of disease modifying therapeutics using α-Syn as a target molecule is supported by recent anti- α-Syn immunotherapy studies (Masliah, Rockenstein et al. 2005, Masliah, Rockenstein et al. 2011). Many approaches are being undertaken in both preclinical and clinical settings as well to find an effective therapy.

Given significant polymorphism among the amyloids, the two major challenges faced in field of protein aggregation are to study the formation of polymorphic (strains) amyloids and their interaction with other amyloid proteins. Such studies are hindered by the lack of structural studies and development of conclusive biomarkers. Interactions between different amyloidogenic proteins could be strain-dependent and may play critical role in disease pathophysiology and phenotypes. The studies performed in this thesis are critical

as they may lead to development of strain-specific antibodies that can be used to study prevention and reversal of behavioral deficits in tauopathy and Synucleinopathies transgenic mouse model. The findings here represent a significant step to elucidate the toxic interplay between α-Syn oligomeric strains and tau altering the aggregation profiles and nature of the amyloid deposits. This could possibly be resulting in the formation of unique aggregates that can cause specific loss of functions of important proteins and impairment of cellular machineries. Insights into the pathogenic interaction between α-Syn and tau will lead to further investigation of their upstream or down-stream interacting proteins that may also have potential roles in disease pathologies. This will lay the groundwork for more successful therapeutic interventions by targeting multiple candidate molecules, such as α-Syn and tau in diseases.

Part of this section has been taken from "Sengupta U, Kayed R (2019) Amyloid oligomer interactions and polymorphisms: disease-relevant distinct assembly of α-synuclein and tau. Neuropsychopharmacology. 44(1):222-223. doi: 10.1038/s41386-018-0204-8".

FUTURE STUDIES

The establishment of distinct recombinant α-Syn oligomeric strains using the physiological inducers in this thesis work has laid the groundwork for investigating in vivo roles of these naturally occurring oligomers. I will perform intracerebrovascular injections of these oligomeric strains as well as brain-derived α-Syn oligomeric strains in wild type animals as well as transgenic animal expressing human α-Syn protein and study the spreading patterns of these oligomeric strains.

The DA- and DHA-induced α-Syn oligomeric strains are being studied for nuclear magnetic resonance (NMR) by our collaborator. Additionally, the cross-seeded aggregated tau strains will be analyzed by CryoEM, which is an effective method to discriminate between amyloid strains. We have initiated this project with one of our collaborators.

The findings from this work enabled us to identify a novel perspective of mechanisms underlying toxicity caused by interacting strains of α-Syn oligomers and tau. The neurotoxic strains of oligomeric α-Syn as well as cross-seeded tau aggregates, identified in this study will serve as candidate molecules for generating specific antibodies and aptamers. Such strain-specific antibodies and aptamers can be used for developing novel therapeutic interventions for the disease at initial stages.

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Vita

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Education

Post Graduate Diploma, 2006
Bioinformatics
IISWBM, Electronics Corporation of India Ltd., India

Master of Science, 2003
Zoology
University of Calcutta, India

Teaching/Mentoring Responsibilities

Mentees/Trainees:

Zachary Watson, Cell Biology Graduate Program, BBSC Rotation, 2018

Jonathan B. Eledge, School of Medicine, Summer Research Program (MSSRP), 2018

Tasneem Hassan, Resident applicant, Dept of Neurology, 2017

Addison Ward, Summer Undergraduate Research Program, 2016, Awarded Best Overall Poster UTMB SURP Symposium

Riddhi Bodani, School of Medicine, Summer Research Program (MSSRP), 2015 & 2016, Awarded MSSRP Best Translational Science Poster Award

Ravi Thakker, School of Medicine, Summer Research Program (MSSRP), 2016

Kelsey English, School of Medicine, Summer Research Program (MSSRP), 2014
Awarded MSSRP Best Neuroscience Poster Award, Sealy Center Forum on Aging Best Neuroscience Poster Award, and National Student Research Forum Best Oral Presentation in Neuroscience

Cody Jeu, School of Medicine, Summer Research Program (MSSRP), 2014

Scott Shaffiei, School of Medicine, Summer Research Program (MSSRP), 2014

Alak Dutta, Cell Biology Graduate Program, BBSC Rotation, 2013

Yembur Ahmad, Visiting student, 2013

Malika Farhed, Research Associate, 2013, Kayed Laboratory

Shashirekha Krishnamurthy, Research Associate, 2012, Kayed Laboratory

Professional Affiliation

2018- Student Member, American Neurological Association

2012- Student member, Society for Neuroscience

Honors and Awards

2019 The George Sealy Research Award in Neurology

2019 Jen Chieh and Katherine Huang Memorial Scholarship (Graduate School for Biomedical Sciences)

2019 2nd place for oral presentation at the 6th Annual Cell Biology Graduate Program Symposium

2018 Rose and Harry Walk Memorial Award (Graduate School for Biomedical Sciences)

2018 Travel award for 143rd annual meeting for American Neurological Association Abstract chosen for Data Blitz presentation at the presidential session for American Neurological association, 2018

2017 Jen Chieh and Katherine Huang Memorial Scholarship (Graduate School for Biomedical Sciences)

2017 2nd place for poster presentation at the 4th Annual Cell Biology Graduate Program Symposium

Publications

Peer-reviewed Journals

1. **Sengupta U**, Puangmalai N, Bhatt N, Garcia S, Zhao Y, Kayed R (2020). Polymorphic α-Synuclein Strains Modified by Dopamine and Docosahexaenoic Acid Interact Differentially with Tau Protein**.** ***Molecular Neurobiology*** (accepted)

2. Montalbano M, McLean S, **Sengupta U**, Puangmalai N, Bhatt N, Ellsworth A, Kayed R (2019). Tau oligomers mediate aggregation of RNA-binding proteins Musashi1 and Musashi2 inducing Lamin alteration. ***Aging Cell.*** e13035. doi: 10.1111/acel.13035

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10. Kolarova M, **Sengupta U**, Bartos A, Ricny J, Kayed R (2017).Tau Oligomers in Sera of Patients with Alzheimer's Disease and Aged Controls. ***J Alzheimers Dis***. 58(2):471-478. doi: 10.3233/JAD-170048.

11. **Sengupta U**, Portelius E, Hansson O, Farmer K, Castillo-Carranza DL, Woltjer R, Zetterberg H, , Galasko O, Blennow K, Kayed R (2017). Tau oligomers in cerebrospinal fluid in Alzheimer's disease. ***Annals of Clinical and Translational Neurology***.

12. Tau Oligomers Associate with Inflammation in the Brain and Retina of Tauopathy Mice and in Neurodegenerative Diseases. Nilson AN, English KC, Gerson JE, Barton Whittle T, Nicolas Crain C, Xue J, **Sengupta U**, Castillo-Carranza DL, Zhang W, Gupta P, Kayed R (2017) ***J Alzheimers Dis***. 55(3):1083-1099. doi: 10.1002/acn3.382. eCollection 2017 Apr.

13. **Sengupta U**, Nilson AN, Kayed R (2016). The Role of Amyloid-β Oligomers in Toxicity, Propagation, and Immunotherapy. ***EbioMedicine***, 6:42-9. doi: 10.1016/j.ebiom.2016.03.035.

14. Gerson JE, Castillo-Carranza DL, **Sengupta U**, Bodani R, Prough DS, DeWitt D, Hawkins BE, Kayed R (2016). Tau oligomers derived from Traumatic Brain Injury

cause cognitive impairment and accelerate onset of pathology in Htau mice. ***J Neurotrauma***. [Epub ahead of print]

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1. **Sengupta U,** Hasan T, Wilcock DM, Kayed R. Tau oligomer-specific antibodies in intravenous immunoglobulins (IVIGs) a potential therapeutic significance in Alzheimer's disease and neurodegenerative tauopathies. In preparation.

2. Gerson JE, **Sengupta U**, Farmer K, Ha Y, Zhang W, & Kayed R. Characterization of novel disease-specific tau oligomeric strains (In preparation).

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1. **Sengupta U**, Carretero-Murillo M, & Kayed R (2018). "Preparation and Characterization of Tau Oligomer Strains". Tau Protein: Methods and Protocols, Vol. 1779, Editor, Einar M. Sigurdsson; 113-146. doi: 10.1007/978-1-4939-7816-8_9.

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