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Alpha Synuclein Oligomers in Human Pathology:

Significance of Nitrative Alpha Synuclein Modifications

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Alpha Synuclein Oligomers in Human Pathology: Significance of Nitrative Alpha Synuclein Modifications

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Alpha Synuclein Oligomers in Human Pathology: Significance of Nitrative Alpha Synuclein Modifications

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Abstract:

Intracellular deposition of fibrillar β-sheet aggregates of αSyn is a pathological hallmark of synucleinopathies such as Parkinson disease (PD) and dementia with Lewy bodies (DLB), which are clinically characterized by motor and cognitive dysfunction. Recent evidence indicates that αSyn oligomer intermediates are the most neurotoxic species. However, the mechanism of action of αSyn oligomers remains elusive. Previous studies in our laboratory showed that αSyn oligomers cause increased intracellular calcium, thus promoting activation of protein phosphatase calcineurin and concomitant decrease in transcription factor pCREB, which disrupts synaptic integrity and memory. This phenomenon is observed *in vitro, ex vivo,* and *in vivo* and results in deficits in LTP, synaptic plasticity, and memory function. On this basis, I tested the hypothesis that similar effects on synaptic signaling can be observed in human brains from DLB

patients. Here I report that in the brains of DLB patients there is an increase in activated calcineurin (CaN), a significant reduction of pCREB, and this is associated with the appearance of αSyn dimers. Furthermore, I determined the direct toxicity as well as the effect on αSyn oligomers of nitrated αSyn, an oxidative stress-related αSyn modification that has been reported to occur in human synucleopathies. My results show that, as compared to unmodified αSyn, nitrated αSyn elicits no increases in intracellular calcium, shorter LTP expression, and no effects on pCREB levels after sixty minute treatment but decreased cell viability after twenty-four hours. Notably, seeding of unmodified αSyn with small amounts of nitrated αSyn resulted in very stable oligomers that were substantially more toxic than non-seeded αSyn oligomers. Overall these results support the idea that increased CaN activity mediates some of the neuro-dysfunctional effects of aggregated αSyn in humans and may thus constitute a viable pharmacological target. These data also suggest that nitrative modifications of small amounts of αSyn, although not particularly toxic per se, may seed unmodified local αSyn to form stable oligomers that possess increased neurotoxicity. This observation may constitute a molecular mechanism linking oxidative/nitrative insult to synucein pathology that could trigger a detrimental cascade towards neurodegeneration.

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Chapter 1: Background

Preface

Lewy Bodies (LB) are intracellular proteinaceous deposits that are composed primarily of aggregated alpha-synuclein (αSyn) and are hallmarks of synucleinopathies such as Parkinson disease (PD) and dementia with Lewy bodies (DLB), two terminal neurodegenerative diseases. The clinical symptoms of these disorders involve motor dysfunction (tremor, bradykinesia and rigidity) and in the case of DLB, also involve cognitive symptoms (delirium, visual hallucinations, delusions) similar to Alzheimer Disease (AD), the most common age-associated dementia. While evidence suggests that aggregation of αSyn into toxic oligomeric assemblies plays a central role in the pathogenesis of these disorders, the underlying molecular events remain elusive. With this goal in mind, this project aims at elucidating two molecular aspects associated with the deleterious effects of αSyn on the central nervous system (CNS):

1. Identifying molecular events in diseased human brain that may mediate the detrimental effects of αSyn oligomers.
2. Identifying posttranslational modifications from oxidative/nitrative stress on αSyn that are associated with toxicity through triggering of synuclein-pathology.

The results from these studies characterize a novel pathway centered on CaN and linking synaptic dysfunction with αSyn oligomers. Furthermore, these results provide a link between oxidative/nitrative stress and synucleinopathies.

Amyloid proteins and amyloid aggregation

Amyloid was erroneously characterized as a starch when it was first discovered based on rudimentary iodine-staining techniques, and thus was given the Latin name *amylum,* meaning starch. However, it is now correctly determined that amyloid is actually a macromolecular aggregate of proteins (Osborne, Butler et al. 1979). Amyloid aggregates have common structural features such as 6–10 nm cross β-sheet structures that are parallel, in-register (Chen, Margittai et al. 2007; Margittai and Langen 2008), and can be identified under cross-polarized light after staining with Congo Red (Morgan, Colombres et al. 2004). Amyloid aggregates form through a seeding/nucleation pathway beginning with the misfolding of a natively structured protein.

The amyloidogenic pathway of protein misfolding and aggregation is not completely understood, but kinetic investigations have implied that the critical phase in amyloidogenesis is the development of protein oligomers acting as seeds to accelerate protein misfolding (Harper and Lansbury 1997), which is the foundation for nucleation-dependent aggregation (Gajdusek 1994). Amyloidogenesis begins with a conformational transition into a β-pleat structure followed by nucleation-dependent aggregation after the protein concentration surpasses a threshold known as the critical concentration (Harper and Lansbury 1997). Disaggregation occurs more often than aggregation between monomers during a lag phase in which oligomers are slowly developed, providing an ordered nucleus/template that catalyzes the further elongation into polymers. This growth phase persists until symmetry between monomers and aggregates is obtained (steady-state phase) (Jarrett and Lansbury 1993). Furthermore, the addition of preformed nuclei (seeds) to amyloid monomers can serve as templates for the aggregation reaction, resulting in elimation of the slow stage of primary nucleation. The aggregation pathway then translates into higher-order oligomers and continues with

aggregation into protofibrils, fibrils, and finally deposits (amyloid pathway shown in Figure 1.1).

The different structures in the amyloidogenic pathway have great morphological diversity as revealed using atomic force microscopy (AFM) and electron microscopy (EM). For example, the smaller molecular weight oligomers of αSyn in synucleinopathies have a spherical appearance with heights ranging 2-5 nm while protofibrils resemble chains of spheres with heights ranging 3-7 nm (Conway, Lee et al. 2000). Oligomeric and protofibrillar intermediates have been shown to be the most toxic species. For instance, *in vitro* studies show that αSyn oligomers, unlike monomers or fibrils, are toxic to neural cells (Demuro, Mina et al. 2005)(Martin et al. recently submitted). Furthermore, unlike higher molecular weight fibrils and Lewy body deposit, naturally occurring αSyn oligomers extracted from the brains of αSyn transgenic mice and PD patients demonstrate a correlation between age and disease severity (Sharon, Bar-Joseph et al. 2003). Indeed, recent evidence has shown that the higher weight aggregates might actually be formed as a protection strategy by sequestering the toxic oligomeric intermediates (Olanow, Perl et al. 2004).

Cellular pathology of oligomers

αSyn oligomers are heterogeneous (dimer, trimer, hexamer, protofibril, etc.) and are similar in structure to other oligomers from several neurodegenerative diseases as demonstrated using EM, AFM, and conformational antibodies, (Kayed, Head et al. 2003; Kayed, Sokolov et al. 2004; Demuro, Mina et al. 2005). One intermediate in the amyloidogenic pathway, annular protofibrils, can readily integrate into the cell membrane, perturbing membrane permeability, and causing ion dyshomeostasis such as increased intracellular Ca^{2+} levels (Lashuel, Hartley et al. 2002; Kayed, Head et al. 2003; Zhu, Li et al. 2003; Demuro, Mina et al. 2005; Glabe and Kayed 2006). Indeed,

AFM studies have revealed protofibrils of wildtype and mutant αSyn to be structurally similar to pore-forming bacterial toxins (Ding, Lee et al. 2002; Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002). These pore-forming bacteria such as *Staphylococcus aureus* and *Clostridium perfringens* can adopt amphipathic β-sheet oligomers with hydrophobic interior and cationic hydrophilic ends that span the lipid bilayer to form pores in the membrane (Butterfield and Lashuel ; Hotze, Heuck et al. 2002). Both charge and hydrophobic interactions can form between membranes and αSyn oligomers as well (van Rooijen, Claessens et al. 2009). Furthermore, annular protofibrils of αSyn induce leakage from cells only of low-molecular mass molecules no larger than 26 Å while the inner diameter of αSyn annular protofibrils, as observed using EM, is 25 Å, inferring membrane destabilition through a pore-like mechanism (Ding, Lee et al. 2002; Volles and Lansbury 2002). Interestingly, the anti-oligomeric antibody A11 inhibits membrane-permeabilizing activity of both amyloid oligomers and pore-forming toxins, suggesting these similar structures elicit toxicity using similar mechanisms (Kayed, Sokolov et al. 2004; Yoshiike, Kayed et al. 2007). The most direct evidence of the pore-forming mechanism of protofibrils was shown by AFM in reconstituted membranes in which the protofibrils form ion-channel-like structures and elicit single ion-channel currents that lead to ion dyshomeostasis (Quist, Doudevski et al. 2005).

Oligomer induced ion dyshomeostasis such as rises in intracellular Ca^{2+} could profoundly affect neuronal function and eventually cause neuronal death (Volles and Lansbury 2003). Consequently, any neuron type affected by aggregating αSyn (Braak, Del Tredici et al. 2003) can suffer the destabilizing action done on the cell membrane, which may be one common event underlying cognitive and motor symptoms in synucleinopathies. This effect of intracellular Ca^{2+} on neuronal dysfunction caused by the toxic αSyn oligomers could be a target for therapeutic intervention; however, it is not

fully understood which players are involved in oligomeric αSyn induced neurotoxicity. One proposed participant is calcineurin (CaN), a Ca^{2+}-sensitive protein phosphatase abundant in the brain and involved in synaptic plasticity and memory function, as being a key mediator of αSyn oligomer induced synaptic dysfunction and cognitive deficits. CaN is highly abundant in the CNS (Polli, Billingsley et al. 1991), is predominantly localized in the synapse (which is shared by αSyn), and has great influence over synaptic plasticity, memory, and cell death.

Calcineurin

The serine-threonine protein phosphatase CaN, also called protein phosphatase 2B (PP2B) or protein phosphatase 3 (PP3), contains two subunits. The catalytic subunit A has three isoforms (α, β, γ), and the first two (α, β) are highly expressed in the brain and enriched in dendritic spines of neurons (Halpain, Hipolito et al. 1998; Sik, Hajos et al. 1998). Even though subunit A is primarily the catalytic subunit, it also contains the autoinhibitory domain, which is located near the carboxy-terminus downstream of the Ca^{2+}/calmodulin binding domain. The autoinhibitory domain binds in the active-site cleft when Ca^{2+}/Calmodulin is absent, inhibiting the enzyme. Activation of the enzyme occurs in response to increases of intracellular Ca^{2+}, when the binding of Ca^{2+}/Calmodulin causes a conformational change in CaN, exposing the active site (Kissinger, Parge et al. 1995). As a consequence of this Ca^{2+}-dependent activation mechanism, CaN is a critical regulator of Ca^{2+} signaling, synaptic plasticity, learning and memory, and cell survival (Winder, Mansuy et al. 1998; White, Sullivan et al. 2000; Josselyn, Kida et al. 2004; Yang, Omori et al. 2004).

Bcl-2 associated death protein (BAD), a mediator of cell death, is one protein activated through the dephosphorylation of CaN (White, Sullivan et al. 2000; Yang,

Omori et al. 2004). CREB is another protein dephosphorylated directly and indirectly (via activation of protein phosphatase-1, PP-1) by CaN (Winder, Mansuy et al. 1998; Nishi, Snyder et al. 1999; Josselyn, Kida et al. 2004), causing dephosphorylation and inactivation of this transcription factor for key genes involved with synaptic plasticity, long term potentiation (LTP, the cellular basis of learning and memory), and learning and memory, such as FOS and Egr-1 (Mansuy, Mayford et al. 1998; Malleret, Haditsch et al. 2001). PP-1 is normally inhibited by dopamine-and-cyclic-AMP-regulated-phosphoprotein-of-MW-32-kDa (DARPP-32), but dephosphorylation of DARPP-32 by CaN attenuates this inhibition, allowing PP-1 to dephosphorylate substrates involved in synaptic plasticity such as CREB (Nishi, Snyder et al. 1999), AMPA receptors, and NMDA receptors (Morishita, Marie et al. 2005). Just as synaptic function, learning, and memory decrease with age, studies have shown CaN to increase in aged brains (Foster, Sharrow et al. 2001). This is notable because aging is also a primary risk factor in the majority of synucleinopathies. (Figure 1.2 shows a putative cell signaling pathway involving αSyn oligomers perturbing membrane permeability, resulting in increased intracellular Ca2+, followed by CaN activation and dephosphorylation of pCREB, leading to synaptic dysfunction.)

Even though increased CaN is associated with synaptic dysfunction and aging, complete elimination of CaN has revealed similar detrimental outcomes on memory, cognition, and performance as shown using the Morris water maze and other behavioral test paradigms (Mansuy, Mayford et al. 1998; Zeng, Chattarji et al. 2001). In fact, studies involving constitutive or inducible genetic manipulation to increase or decrease CaN have shown contrasting results (Mansuy, Mayford et al. 1998; Malleret, Haditsch et al. 2001; Zeng, Chattarji et al. 2001; Sharma, Bagnall et al. 2003). These discrepancies can be explained by differences in the degree of CaN inhibition. For example, transgenic

animals expressing the CaN autoinhibitory peptide only inhibit 35-40% of CaN whereas a CaN genetic knockout model completely inhibits CaN. This variability could also be caused by other factors such as kinase dysfunction which are known to be positive modulators of learning and memory (CaMKII, CaMKIV, PKC) and are also mediated by Ca^{2+}/Calmodulin, the deciphering switch between phosphatase or kinase activation (Wu, Deisseroth et al. 2001; Mansuy 2003). In view of the fact that both LTP and long-term depression (LTD) are generated through both an increase in intracellular Ca^{2+} and binding to calmodulin seems counter intuitive; however, calmodulin's role has shown to be the bidirectional mediator in synaptic plasticity by activating either phosphatases or kinases depending on calcium concentration (Stefan, Edelstein et al. 2008). It is known that CaN has an increased affinity for calmodulin and is activated at lower calcium concentrations than kinases (Stefan, Edelstein et al. 2008). This is complemented by studies showing high-frequency stimulation results in a Ca^{2+} influx threshold high enough to trigger LTP whereas low-frequency stimulation elicits low concentrations of Ca^{2+} triggering only LTD (Bear 1995; Ni and Chen 2008), demonstrating that low intracellular calcium influx during LTD would allow binding of CaN over kinases. In addition to CaN having increased sensitivity to Ca^{2+}, studies have shown that activated CaN decreases the amount of available calmodulin by dephosphorylating proteins that then bind and suppress calmodulin, such as neuromodulin and neurogranin (Liu and Storm 1989; Seki, Chen et al. 1995). Further evidence of CaN-induced calmodulin suppression is observed decrease in levels of phosphorylated neuromodulin and neurogranin during LTD (Ramakers, Heinen et al. 2000), revealing another link between CaN, LTD, and synaptic dysfunction.

Protein misfolding diseases

Protein misfolding diseases (PMD) encompass a variety of disorders that have a similar mechanism that involves a native monomeric protein structural rearranging to a β-sheet rich ordered conformation (Makin and Serpell 2002), and neurodegenerative diseases (ND) are a subcategory of PMD. These neurological disorders are awfully debilitating, affecting movement and/or cognition. This group of diseases constitute clinically diverse disorders including Alzheimer's disease (AD, amyloid-beta is the amyloid), Synucleinopathies such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (αSyn is the amyloid), amyotrophic lateral sclerosis (ALS, SOD1 is the amyloid), Huntington's disease (HD, huntingtin is the amyloid), and transmissible spongiform encephalopathies (TSE, prion is the amyloid) (Martin 2009). Even though these disorders are very diverse clinically, they share a common mechanism of molecular pathogenesis which includes cerebral deposition of insoluble protein deposits, termed amyloid. However, the protein sequence of the misfolded aggregates and the distinct brain regions targeted by these diverse aggregated proteins are different in each of these diseases, leading to clinical differences.

Synucleinopathies

Synucleinopathies comprise an array of neurodegenerative disorders characterized by Lewy Bodies which are insoluble aggregates containing predominantly αSyn protein located in the cytoplasm of neurons and glia. The two most common synucleinopathies are PD and DLB, representing two extremes of motor and cognitive dysfunction, respectively. Even though common clinical features in PD involve motor deficits (bradykinesia, resting tremor, rigidity and postural instability), the term Parkinson

Disease Dementia (PDD) is given to cases in which cognitive impairment is observed in the later stages of PD (Emre 2003). On the other hand, the common clinical characteristics of DLB are cognitive deficits (delirium, visual hallucinations, delusions) while some cases have secondary parkinsonism features; however, the majority of DLB cases develop motor deficits at later stages of the disease (Burn and McKeith 2003). As described, PDD and DLB terminology are derived from the clinical timing of dementia symptoms relative to motor impairments. For example, DLB is described as the development of dementia within one year or less while PDD is a term given when dementia forms over a year after the motor symptoms (McKeith, Galasko et al. 1996). Nevertheless, even though the clinical symptoms in synucleinopathies are distinct at various time-periods during disease, the common thread in the family of synucleinopathies is the misfolding and aggregation of the protein, αSyn.

Further support of αSyn as the etiological player in synucleinopathies were the discoveries of three missense mutations (A53T, A30P, and E46K) in the αSyn protein that are linked to rare forms of familial PD (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998; Zarranz, Alegre et al. 2004). Moreover, αSyn is the primary protein component of the lesions (Lewy Bodies) in brains of patients with either PD or DLB; however, the brain regions affected are distinct (Baba, Nakajo et al. 1998). PD synucleinopathies are most prevalent in the substantia nigra with loss of dopaminergic neurons while DLB has most pathogenesis in the basal forebrain with loss of primarily cholinergic neurons (Duda, Giasson et al. 2002). Pathological progression of DLB begins in layers V-VI and progresses into layer III, then finally layer II (Marui, Iseki et al. 2002); however, even though Lewy Bodies are primarily in the basal forebrain in DLB, Lewy body pathology is frequently seen in limbic regions as well (Hurtig, Trojanowski et al. 2000).

Since synucleinopathies are associated with the loss of dopaminergic and cholinergic neurons, the development of dopaminergic replacement therapies (Savitt, Dawson et al. 2006) and acetylcholinesterase inhibitors (Thomas, Burn et al. 2005) have been used as therapeutics and have shown to provide relief of symptoms, albeit only temporary. In view of the fact that these therapeutics are aimed only at increasing neurotransmitter availability, the cause of synaptic dysfunction, αSyn, is overlooked (McNaught and Olanow 2006).

αSyn (structure and physiological function)

αSyn is a randomly folded fourteen-kDa protein composed of three domains: the amphipathic amino-terminus, the hydrophobic core, and the acidic carboxy-terminus (αSyn structure shown in Figure 1.3). The amino-terminus contains seven 11-amino-acid imperfect repeats containing the consensus motif KTKEGV (except one, which is KAKEGV) where αSyn interacts with membrane phospholipids using an amphipathic α-helical conformation (Jo, McLaurin et al. 2000). The middle domain is composed of the hydrophobic core and participates in the formation of β-sheet interactions during amyloid aggregation. Interestingly, the amino acid sequence of αSyn's hydrophobic core is termed non-amyloid-β component (NAC) because this region is also found in amyloid-β plaques from AD patients (Weinreb, Zhen et al. 1996). The carboxy-terminus makes up a disordered conformation and is rich in acidic residues.

Although αSyn is primarily an intrinsically disordered structure, it tends to self-associate through two interactions: 1. charge attraction between the negatively charged carboxy-terminus collapsing around the net positive charge of the amino-terminus, 2. hydrophobic interactions between the hydrophobic core and a hydrophobic cluster region in the carboxy-terminus (Bertoncini, Jung et al. 2005; Dedmon, Lindorff-Larsen et

al. 2005). Experimental evidence has revealed this conformation using small angle x-ray scattering showing the gyration radius of αSyn being ~40 Å, which is larger than a globular protein of similar size but smaller than an unfolded protein of similar size (Uversky, Li et al. 2001), demonstrating a partially folded conformation. Also, nuclear magnetic resonance (NMR) studies have shown the existence of intra-molecular interactions between the carboxy tail and residues 30-100 (Bertoncini, Jung et al. 2005). Agents that release these intra-molecular interactions cause an increase in aggregation, and it is therefore thought that these intra-molecular interactions could be a protection strategy against toxic aggregation (Fernandez, Hoyer et al. 2004; Bertoncini, Jung et al. 2005).

αSyn is located in the presynaptic terminal as the former section of the name "synuclein" implies, but not in the nucleus as first described (Maroteaux, Campanelli et al. 1988; Murphy, Rueter et al. 2000). Studies have shown αSyn's normal function being involved with synaptic vesicle regulation (Murphy, Rueter et al. 2000; Cabin, Shimazu et al. 2002; Larsen, Schmitz et al. 2006), specifically negative regulation of dopamine neurotransmission (Abeliovich, Schmitz et al. 2000) by inhibiting phospholipase D2 (Jenco, Rawlingson et al. 1998; Ahn, Rhim et al. 2002; Outeiro and Lindquist 2003; Payton, Perrin et al. 2004), a membrane-associated enzyme involved in the hydrolysis of phosphatidylcholine which influences cytoskeletal regulation and endocytosis (Brown, Henage et al. 2007). However, inactivation of the αSyn gene does not lead to phenotypic neurodegeneration. Therefore, αSyn loss-of-function most likely does not play a primary role in neurodegeneration (Abeliovich, Schmitz et al. 2000). Instead, a gain-of-function leading to the toxic misfolding and aggregation of αSyn is a more likely etiological component in synucleinopathies.

αSyn (pathological misfolding/function)

αSyn amyloidogenic aggregation occurs primarily through the hydrophobic core but also through the repeats in the amino-terminal half (Serpell, Berriman et al. 2000). The three missense mutations seen in familial PD (A30P, E46K, and A53T) are all located in the amino-terminus region, close to the hydrophobic core. These mutations promote oligomerization using different mechanisms: the A30P mutation decreases helical formation in the amino-terminus, the A53T mutation causes an increase in β-sheet conformation around the site of mutation, and the E46K mutation causes mild helical rearrangement (Bussell and Eliezer 2001; Fredenburg, Rospigliosi et al. 2007). Furthermore, both A30P and A53T mutations interfere with the intra-molecular interactions between the carboxy-terminus and amino-terminus (Bertoncini, Jung et al. 2005). Even though the mutations have variable effects on αSyn, they all promote oligomerization, suggesting oligomerization as a key initiator of neurodegeneration. Furthermore, A53T and A30P mutants have significantly greater membrane destabilization effects, suggesting this mechanism of toxicity by αSyn protofibrils as being a key event in PD pathology (Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002; Volles and Lansbury 2002).

Another indicator of intramolecular influences on aggregation kinetics are the different effects after calpain truncation. For example, calpain truncation on preformed αSyn oligomers cleaves oligomers after amino acids 57, 73, 74, and 83, interfering with the fibrillogenic hydrophobic core and inhibiting fibrillation. Conversely, calpain truncation of preformed αSyn fibrils cleaves after residues 114 and 122, accelerating fibrillation (Hodara, Norris et al. 2004; Mishizen-Eberz, Norris et al. 2005). Calpain truncation differences on oligomeric versus fibrillar conformations is a clear demonstration of the conformational differences amongst the different aggregates and reveals that the

hydrophobic core is necessary for fibrillation while the carboxy-terminus is important for fibril inhibition. Studies have shown carboxy-terminal truncations of αSyn leads to increased fibrillation (Li, West et al. 2005), promote nucleation (Hoyer, Cherny et al. 2004), and even seed aggregation of full-length αSyn (Li, West et al. 2005). This is because the carboxy-terminus normally inhibits fibrillation by interacting with the amyloidogenic hydrophobic core.

αSyn (intracellular vs extracellular)

Classically, αSyn aggregates have been described as being intracellular, consistent with αSyn's most abundant localization. However, recent evidence has revealed αSyn aggregates also being extracellularly. For example, evidence to show αSyn's ability to bypass cell membranes is shown by αSyn's presence in the CSF and plasma of PD patients (Borghi, Marchese et al. 2000; El-Agnaf, Salem et al. 2003) as well as studies showing cultured neurons releasing αSyn in the medium (Lee, Patel et al. 2005). Furthermore, fetal tissue grafted into the CNS of PD patients eventually developing αSyn aggregates (Kordower, Chu et al. 2008; Li, Englund et al. 2008), suggesting the possibility of inter-neuronal transmission of extracellular αSyn aggregates (Brundin, Li et al. 2008).

αSyn (posttranslational modifications and pathological significance with emphasis on nitration)

Although the spotlight shines on oligomers as being the most toxic amyloid aggregate in neurodegenerative diseases, recent evidence has shown that not all oligomers share similar characteristics. For example, manipulations on αSyn such as posttranslational modifications can change αSyn's secondary and tertiary structure and hence, function and toxicity. These influential manipulations demonstrate that factors

such as amino acid sequence intertwined with environmental conditions determine which pathway this amyloid will pursue leading to one of three aggregates: amorphous, on-pathway, or off-pathway (the three aggregates are illustrated in Figure 1.4) (Serpell, Sunde et al. 1997; Kodali and Wetzel 2007). Amorphous aggregates are formed spontaneously without any conformational guidelines, causing the protein to immediately precipitate out of solution (Uversky). On the other hand, pathological on-pathway amyloid aggregates form through specific conditions such as increased concentration of αSyn, agitation, increase in temperature, increase in salt, or decrease in pH to cause a monomer to misfold and aggregate through β-sheet interactions to form a nucleus/template during this rate-limiting step termed lag phase (Wetzel, Shivaprasad et al. 2007). These earlier formed αSyn oligomeric species are transient and have high dissociation rates, but once this nucleation-dependent aggregation reaches critical stage in which the association rate exceeds the dissociation rate, amyloid aggregation then increases exponentially and plateaus (Bhak, Choe et al. 2009). αSyn oligomers formed on this pathway disappear as 7-13 nm fibrils begin to form, demonstrating oligomer formation followed by fibril formation on the same pathway (Dobson 1999). However, some oligomeric species are derived from an off-pathway aggregation without a nucleation-dependent lag phase because lack of a high dissociation rate which by overstepping this rate-limiting step leads to a quicker formation of the oligomers (Zhou, Long et al. ; Yamin, Uversky et al. 2003). In addition, because such off-pathway oligomers have increased stability, their further aggregation is greatly reduced and thus formation of fibrils significantly inhibited. Off-pathway oligomers are created through various manipulations to the αSyn protein such as interactions with nicotine or flavinoids, but the present study focuses on posttranslational modifications caused by nitrative and oxidative environments because of the significance of free radical stress to synucleopathic diseases as outlined below.

Under mild oxidizing conditions, methionine residues 1, 5, 116, and 127 are oxidized to methionine sulfoxide. Methionine oxidation causes stable off-pathway oligomers by two mechanisms: 1. stabilizing the intra-molecular hydrophobic interactions between the hydrophobic core and the hydrophobic cluster in the carboxy-terminus and 2. long-range electrostatic interactions between the acidic carboxy-terminus and net positive charge of the amino-terminus (Zhou, Long et al.). Even though methionine oxidation creates stable oligomers that inhibit fibril formation, these oligomers caused during mild oxidization have been shown to be nontoxic to dopaminergic neurons (Zhou, Long et al.).

Under stronger oxidizing conditions such as occurring during neuroinflammation (Gao, Kotzbauer et al. 2008), reactive free radicals like superoxide and the normally neuroprotective nitric oxide are pathologically increased (Huie and Padmaja 1993). During such oxidative and nitrative stress, superoxide and nitric oxide form peroxynitrite, and any of these three reactive species can then extract an electron from tyrosine residues to form very reactive tyrosyl radicals. These tyrosyl radicals then react with nitrative species again and form 3-nitrotyrosine or react with another tyrosyl radical and form *o'o* dityrosine (chemical reaction shown in Figure 1.5) (Koppenol, Moreno et al. 1992; Pfeiffer, Schmidt et al. 2000). Under physiological conditions, dityrosine crosslinking is favored over nitrotyrosine formation because there is decreased availability of nitrogen and oxygen free radicals in comparison to tyrosyl radicals as a result of nitrogen and oxygen free radicals frequently reacting with each other (Pfeiffer, Schmidt et al. 2000).

αSyn contains four Tyr located at residues 39, 125, 133, and 136, and the reactive affinities of these Tyr residues to nitration differ substantially (Bertoncini, Jung et al. 2005; Dedmon, Lindorff-Larsen et al. 2005). For instance, the three Tyr residues in

the Asp and Glu rich carboxy-terminus are highly reactive to nitration due to its high flexibility and polarity (Clayton and George 1999). Tyr 39, on the other hand, lies in the amino-terminus close to the more rigid hydrophobic core and also where lipid membrane-associated α-helices are located, so it is less exposed to solvent, making this Tyr the least reactive to nitration (Hashimoto, Rockenstein et al. 2001; Ruf, Lutz et al. 2008). With this in mind, αSyn in the collapsed formation and nitrative effects primarily on carboxy Tyr would sterically hinder more than two Tyr to react (Ruf, Lutz et al. 2008), favoring dimers. Once sufficient population of covalently formed dimers accumulate, they would form higher molecular weight even-numbered oligomers (Conformation of dityrosine crosslinking of two αSyn monomers shown in Figure 1.6) (Souza, Giasson et al. 2000; Takahashi, Yamashita et al. 2002; Ruf, Lutz et al. 2008).

Oxidative/Nitrative stress and Synucleinopathies

Regardless of their different reactivity, nitration of any of the four Tyr residues of αSyn causes a decrease in helical membrane binding, implying Tyr nitration develops an allosteric structural change on αSyn that can even ameliorate αSyn's normal function on vesicle trafficking (Sevcsik, Trexler et al. ; Hodara, Norris et al. 2004). Aside from nitration effects on αSyn's normal functioning mentioned above, studies on nitration effects on neurodegeneration have been sparse and conflicting. For instance, one recent study has shown using an αSyn fused to an internalization signaling peptide that intracellular nitrated αSyn is more toxic than intracellular non-nitrated αSyn on dopaminergic neurons (Yu, Xu et al.); however, the same authors pointed out that the toxicity was diminished when the internalization tag was removed, so the mechanism of this toxicity doesn't seem to involve membrane instability. Indeed, even though αSyn's most commonly-known mechanism of toxicity is through membrane destabilization (Lashuel, Hartley et al. 2002; Volles and Lansbury 2003), not all αSyn oligomers share

this characteristic (Uversky ; Fink 2006). Nonetheless, other studies have shown nitrative stress as being the mediator between the synergism of environmental stimuli (such as inflammatory-stimulating agents or herbicides) and Tg αSyn animal models (Peng, Oo et al. ; Yu, Xu et al. ; Gao, Kotzbauer et al. 2008). However, since nitrated αSyn oligomers are not β-sheet amyloid aggregates but instead are formed through covalent interactions, the mechanism of nitrated αSyn toxicity may differ from amyloid aggregates and remains unclear.

Much evidence has revealed an association between nitrative/oxidative stress and synucleinopathies. For instance, nitrative stress and synucleinopathy incidences increase with age and both elicit insult on the post-mitotic neurons (Choi, Zhang et al.). Furthermore, it is thought dopaminergic neurons are most susceptible during PD because of the oxidative environment created through dopamine metabolism (Dauer and Przedborski 2003). Moreover, oxidative stress and nitration have also been observed on Lewy body deposits in postmortem PD and DLB brains (Duda, Giasson et al. 2000; Giasson, Duda et al. 2000; Gomez-Tortosa, Gonzalo et al. 2002; Dalfo, Portero-Otin et al. 2005). Despite the association between nitrated αSyn with neurodegeneration in synucleinopathies, the extent of this association is not fully understood.

One fundamental difference between nitrated and non-nitrated αSyn is the covalent interaction during dityrosine crosslinking occurring in the former as compared to nucleation-dependent β-sheet aggregation that characterizes the latter. Indeed, aggregation of nitrated αSyn follows an off-pathway oligomerization that does not lead to fibril formation (Souza, Giasson et al. 2000; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). Moreover, dityrosine crosslinking of nitrated αSyn leads to stable oligomers that are resistant to chaotropic treatments (Souza, Giasson et al. 2000; Norris, Giasson et al. 2003; Uversky, Yamin et al. 2005). Importantly, along with dimer formation,

dityrosine crosslinking can crosslink preformed aggregates as well (Souza, Giasson et al. 2000; Norris, Giasson et al. 2003; Ruf, Lutz et al. 2008). Nitration on preformed aggregates, however, is structurally different from nitration of monomers because aggregates are already in a stacked conformation with the tyrosines freely exposed to solvent (Ruf, Lutz et al. 2008). This explains why nitration on Lewy bodies is observed in postmortem tissue of PD and DLB patients as a consequence of the nitation reaction most likely occurring on already formed aggregates. Nonetheless, no matter what species of αSyn is subject to nitrative stress, covalent crosslinking creates increasingly more stable aggregates.

The increased stability of nitrated oligomers indicates that the secondary structures are different from the non-modified oligomers. However, nitrated and non-nitrated αSyn can still influence each other's aggregation pathway as studies have shown that even substoichiometric amounts of the heterogeneous oligomers created through oxidative and nitrative modifications leads to fibril inhibition of non-modified αSyn (Zhou, Long et al. ; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). Conversely, other studies showed opposing effects after isolation using size-exclusion-chromatography (SEC) of nitrated αSyn monomers and dimers that can actually increase fibrillation by acting as seeds and incorporating into fibrils (Gao, Zhang et al. ; Hodara, Norris et al. 2004). The opposing effects on αSyn aggregation from the different nitrated αSyn species suggests differences in rate constants amongst even the oligomeric species of nitrated αSyn. Moreover, the influences of the nitrated αSyn on αSyn implies that the off- and on-pathways are competitive with each other, and the off-pathway aggregation because of its faster kinetics depletes the concentration of available αSyn to reach critical concentration for on-pathway fibrillation. These substoichiometric effects of nitrated αSyn on αSyn are notable because only a fraction of

αSyn is nitrated during nitrative stress (Hodara, Norris et al. 2004). This suggests that even though not all of αSyn is nitrated during nitrative stress, the fraction that is has a dramatic impact on the unmodified αSyn structure and toxicity due to competitive rate constants, revealing an indirect association of nitrative stress on neurodegeneration based on seeding of αSyn toxic aggregates by trace amounts of nitrated αSyn.

Oxidative stress of other molecules, such as lipids, has also been shown to have an indirect effect on αSyn (Conway, Rochet et al. 2001; Qin, Hu et al. 2007; Choi, Liu et al. 2009; Parihar, Parihar et al. 2009). However, the effects of these oxidized molecules on αSyn are conflicting. For example, one product of lipid oxidation, 4-hydroxyl-2-nonenal (HNE), can form covalent lipid-protein adducts with αSyn. This leads to a conformational change and formation of stable oligomers that are significantly more compact than unmodified αSyn, inhibit fibril formation, and cause marked neurotoxicity on primary mesencephalic cultures (Trostchansky, Lind et al. 2006; Qin, Hu et al. 2007; Smith and Cass 2007; Trostchansky and Rubbo 2007). On the other hand, studies have shown that cholesterol oxidation causes increased fibrillation of αSyn and induces its dissociation from cell membranes (Bosco, Fowler et al. 2006). The interaction of oxidized lipids and αSyn demonstrates another potentially pathologic process by which αSyn interacts with membranes; namely, not only does αSyn interact with anionic lipid head groups on membranes through the basic side-chain α-helices in the amino-terminus during normal function and destabilizes membranes during pathological events such as β-sheet aggregation, but αSyn can also interact with oxidized membranes resulting in pathological aggregates.

Overall hypothesis and scope of my research

I hypothesize that: 1. αSyn oligomers have a significant association with CaN activation and pCREB dephosphorylation in human DLB neuropathology, and 2. Nitrative modifications on αSyn potentiates toxicity on non-nitrated αSyn. The scope of this research is to test these hypotheses by pursuing the specific aims of 1. Identifying molecular events in DLB human brains that are not seen in non-diseased individuals but instead correlate to αSyn oligomers and 2. Identifying posttranslational effects on αSyn caused by nitration.

FIGURES

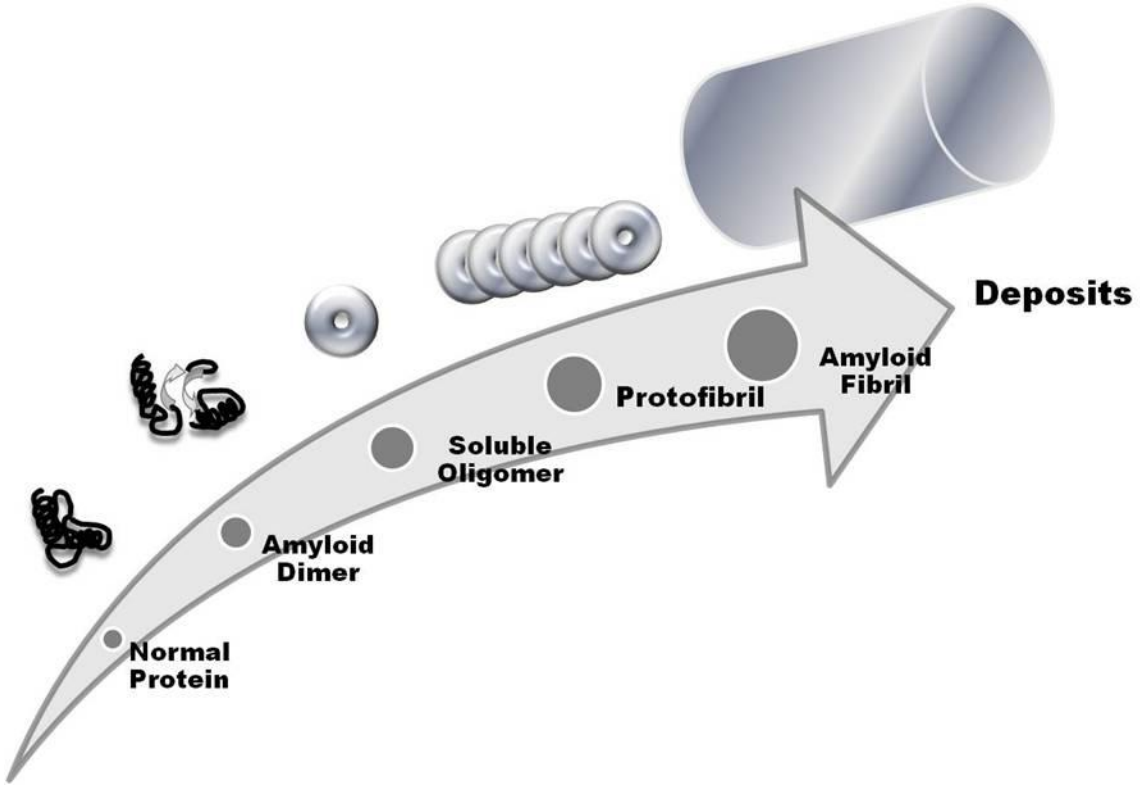


Figure 1.1 Amyloid Aggregation Pathway. Amyloid aggregation pathway starting with a normal protein misfolding into β-sheet conformation, causing nucleation-dependent

aggregation into dimers, higher molecular weight oligomers, protofibrils, and finally deposits.

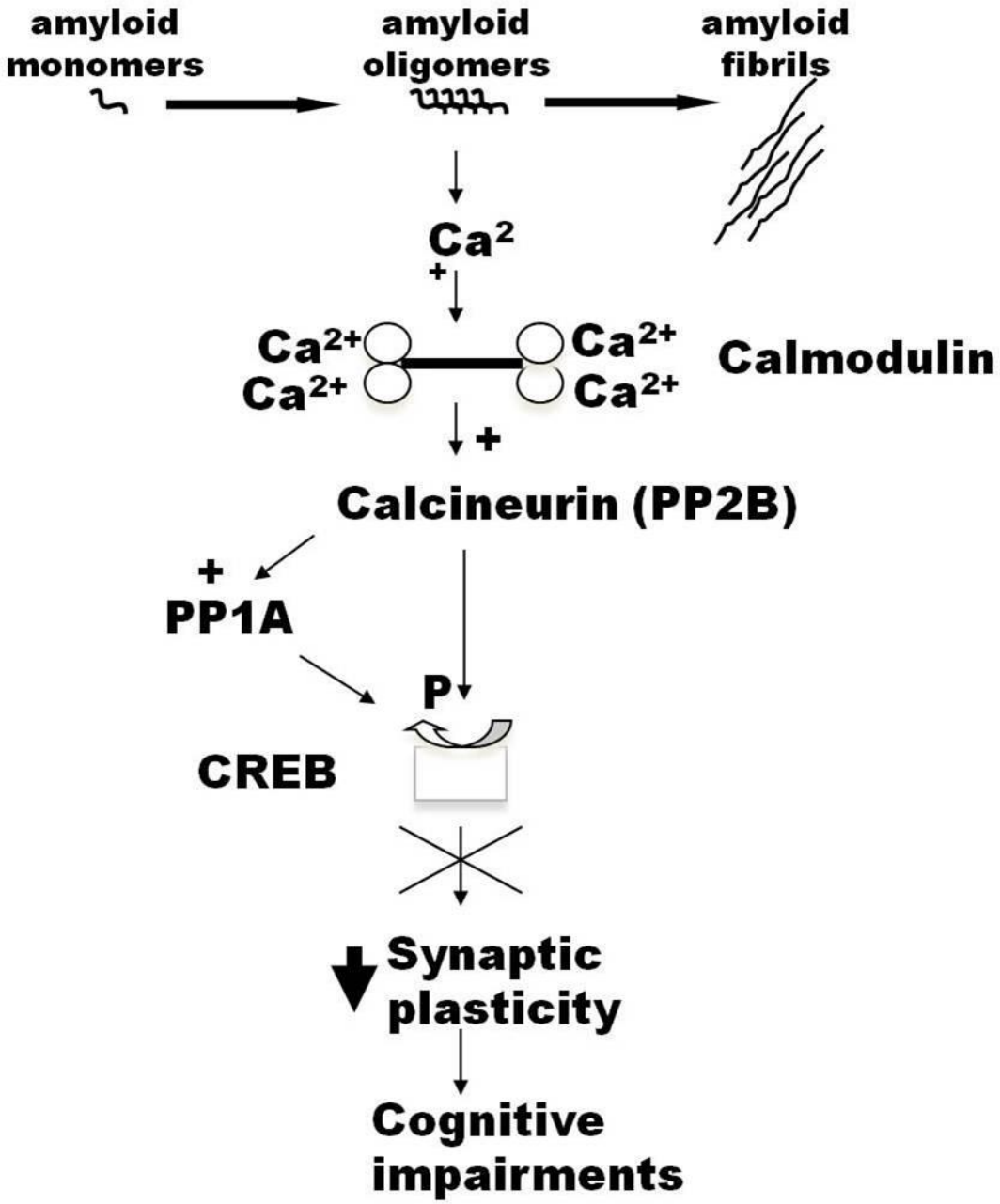


Figure 1.2 Schematic illustrating the putative role of calcineurin in mediating the synaptic and apoptotic consequences of oligomeric αSyn in the CNS. During the amyloidogenic process of misfolded αSyn, soluble oligomeric intermediates may be formed. Such oligomeric

intermediates increase intracellular Ca2+ levels, possibly through cell membrane destabilization. Increased intracellular Ca2+ promoted by oligomeric αSyn induces the activity of the Ca2+/calmodulin-dependent phosphatase CaN. Active CaN dephosphorylates/*inactivates* CREB and DARPP-32, thus leading to impaired synaptic plasticity that may reversibly cause cognitive, behavioral and even motor deficits in synucleinopathies.

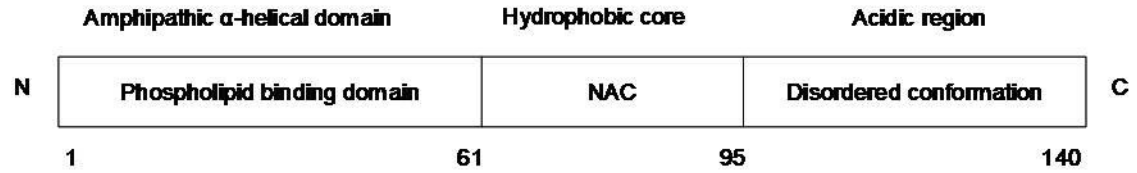


Figure 1.3 αSyn structure composed of three domains. the amphipathic α-helical amino-terminus known to bind phospholipids, the hydrophobic core termed non-amyloid-β component (NAC), and disordered and acidic carboxy-terminus.

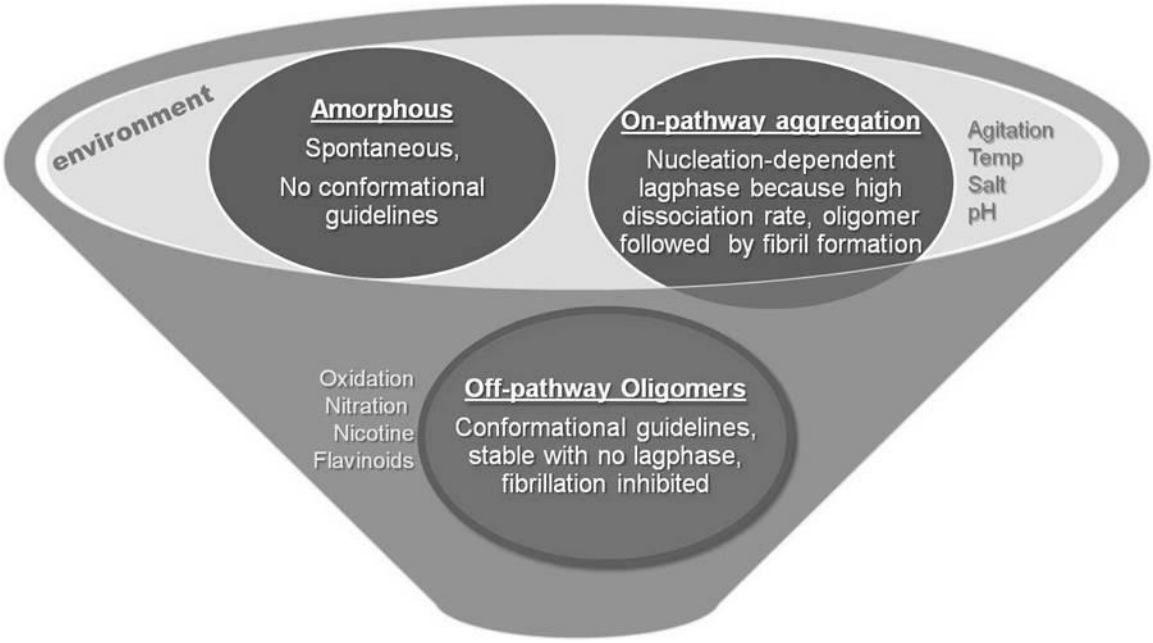


Figure 1.4 Three different aggregates of αSyn depending on the environment. The amorphous aggregate is formed spontaneously without any environmental influences, so there are no conformational guidelines. The on-pathway aggregation is nucleation-

dependent with a lagphase because the early formed aggregates have a high dissociation rate; however, once association rate exceeds dissociation, aggregation becomes exponential and plateaus. This aggregation is termed on-pathway because oligomer formation is followed by fibril formation with disappearance of earlier-formed oligomers. Known environmental factors that influence on-pathway aggregates are increased concentration of αSyn, increased temperature, increased salt, or decreased pH. Finally, off-pathway oligomers have the greatest environmental influence, such as oxidation, nitration, nicotine, or flavinoids. Off-pathway oligomers inhibit fibril formation so are off-pathway. Also, because there is not a high dissociation rate of these early formed oligomers, there is no lagphase.

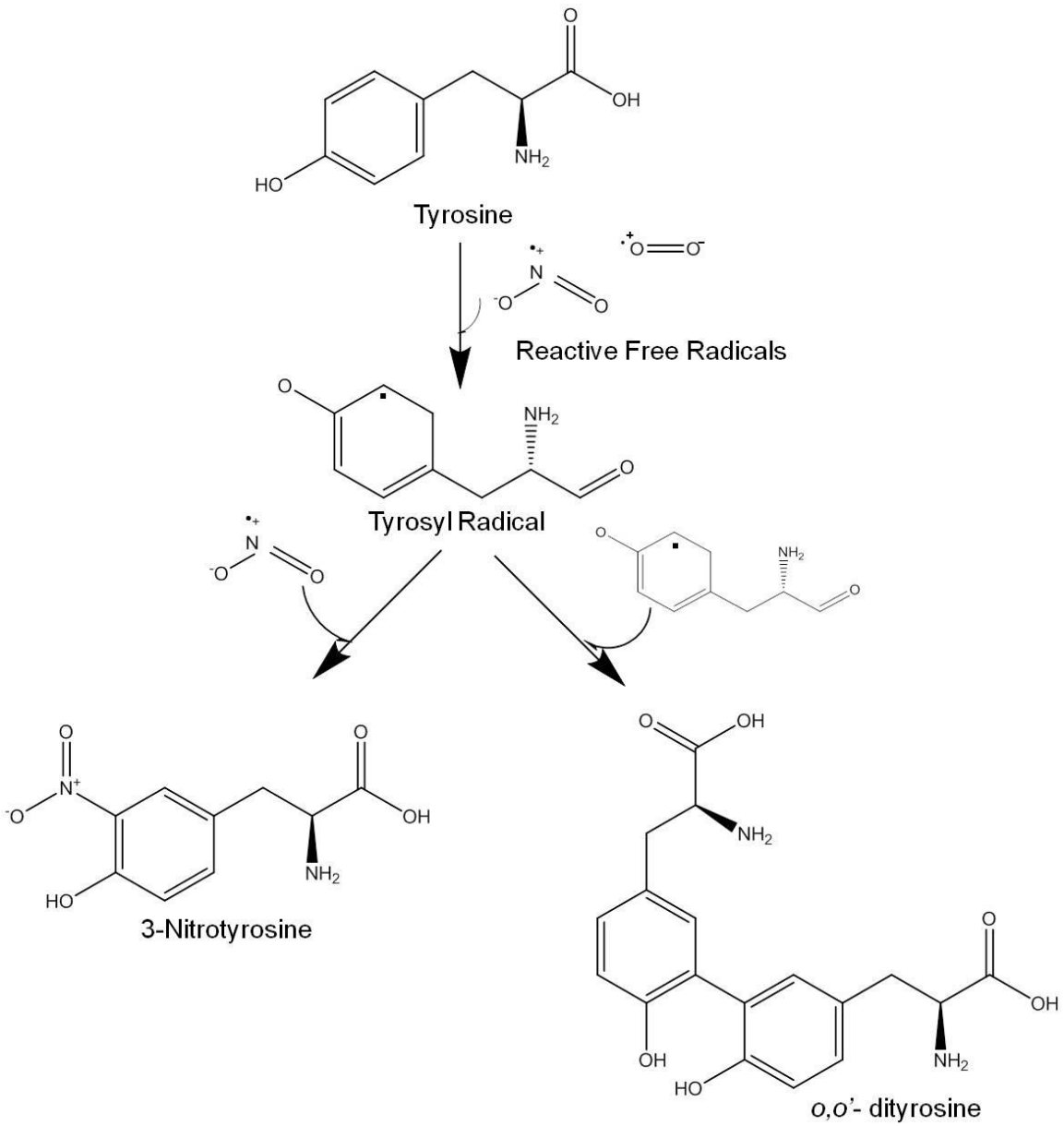


Figure 1.5 Nitration on Tyr residues is a two-step process. First, reactive species extract an electron from tyrosine, causing a highly reactive tyrosyl radical. The tyrosyl radical either reacts with another nitrative radical to form 3-nitrotyrosine or reacts with another tyrosyl radical to form *o'o*-dityrosine. (Pfeiffer, Schmidt et al. 2000)

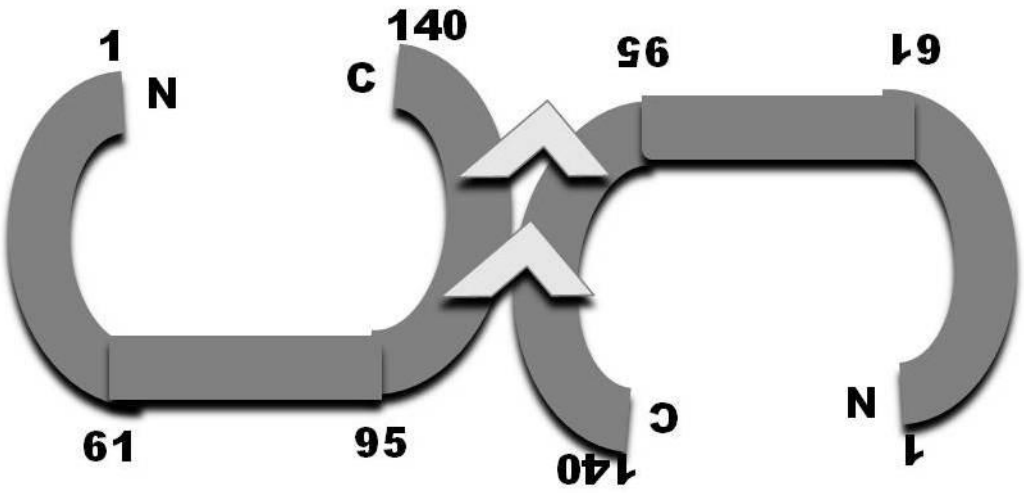


Figure 1.6 Proposed dityrosine crosslinking of αSyn monomers. Nitration on the most reactive tyrosines in the carboxy-terminus would cause steric hindrance for more than two tyrosines to react.

Chapter 2: Methods

Animals

Mice on a C57BL/6 background were used for ICV experiments. Male Sprague Dawley rats (150-250 g) were employed for brain slice preparations. Animals were maintained at the UTMB vivarium under USDA standards (12 hr light/dark cycle, food and water *ad libitum*).

At the end of each experiment, animals were sacrificed by exposure to halothane vapors followed by decapitation. Brains were rapidly removed, and either frozen whole (for immunohistochemistry), or dissected into individual areas including hippocampus, cerebellum, frontal cortex, and occipital cortex, then stored at -80°C until further biochemical analyses were performed.

Acetonitrile/HFIP treatment

5 µl of 90 µM αSyn, NαSyn monomer, and NαSyn oligomer were individually incubated in 250 µl of 50% acetonitrile for 10 minutes at RT followed by overnight lyophilization. These samples were then resuspended in 67 µl HFIP and incubated for 15 minutes at RT and lyophilized overnight. Water and sample buffer were then added followed immediatey by western blot analysis.

AFM

For imaging, 5 µl of ~300 µg/ml of αSyn was adsorbed onto a freshly cleaved mica surface and dried under liquid nitrogen. Samples were imaged in air with a 'home-built' AFM using a Nanotec Scanning Probe Microscope Control System (Dulcinea, Nanotec Electronica) operating in tapping mode, using MSNL cantilevers (0.1N/m, Veeco). Tapping amplitudes were 10-20 nm at the cantilever resonance frequency in air (30-50 kHz). Images were acquired at rates of 60 seconds per image (256 pixels, 2000 nm). Image processing was performed with WSxM software, and standard image processing consisted of flattening and plane subtraction.

Ca^{2+} imaging

SY5Y cells grown on poly-D-lysine coated coverslips were loaded with Fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Slides were then mounted in a stage containing 1mL of serum-free media. Ca^{2+} imaging was performed on an inverted Nikon Eclipse TE200 microscope with a Nikon S Fluor

20X, 0.75 numerical aperture objective at the Optical Microscopy Core, UTMB-Galveston. The samples were excited with a DG4/DG5 xenon source of illumination at two different wavelengths, 340nm and 380 nm respectively controlled by MetaFluor software (Universal Imaging, Dowingtwon, PA, USA). The emission was collected using a 71000av2 filter cube from Chroma Technology Corp (Bellows Fall, VT). equipped with a Fura-2 emitter D510/80m.

The experiment was performed at room temperature. After recording the baseline at t = 5 min, αSyn was added. 20 μM of ionomycin was added as a positive control. Ratiometric measurements and calculations were done using MetaFluor software. The trace shown is the 340/380 ratio averaged response of 30 cells after subtraction of background.

Cell culture

Cultured human neuroblastoma SY5Y cells from American Type Culture Collection (ATCC, Manassas, VA) were maintained at 37 °C in a humidified cell incubator under 5% CO_2 atmosphere in T-125 culture flasks containing 10 mL of DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO). When cultures reached confluence, cells were dislodged using trypsin and divided into two flasks.

Cell Viability assay MTS

This colorimetric assay can be used for determining the number of viable cells in cell growth and cytotoxicity assays through bioreduction by the electron carrier on cells.

Cultured cells were assayed using a commercially available kit (Thermo Fisher) according to manufacturer's instructions.

Data Collection and Statistical Analysis

For Immunohistochemical analyses, equal areas of merged raw digital images were selected from the cortical regions and measured for intensity levels using ImageJ. Microsoft Excel was used for graph production and SPSS Statistics was used for data analysis employing One-way analysis of variance (ANOVA) followed by the Fisher's Least Significant Difference (LSD) test for multiple comparisons. A resulting p value less than 5% ($p<0.05$) was considered statistically significant. Cumulative data are represented as mean $\pm$ S.E.M.

Ex vivo rat brain slices

According to IACUC-approved protocols, rats were decapitated, the brain was quickly removed and blocked in cold (4 °C) artificial cerebrospinal fluid (aCSF) prepared as follows: 117 mM NaCl, 4.7 mM KCl, 1.2 mM Na_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, oxygenated and equilibrated to pH 7.4 with 95% O_2/5% CO_2. 500 μm-thick hippocampal brain slices were prepared using a Vibroslice (Camden Instruments, London, UK) and equilibrated in aCSF at room temperature for 30 min prior to the addition of test compounds. At the end of the experiments, slices were rapidly frozen in liquid nitrogen and stored at -80 °C until further analyses were performed

Human Tissue

Frozen frontal lobe tissue was obtained through a Materials Transfer Agreement with the Oregon Brain Bank at Oregon Health and Science University in Portland (Table 2.1). Brains are donated by subjects enrolled in studies at the NIH-OHSU Layton Aging and AD center. These cases have post-mortem intervals (PMI) ranging from 3-48 hours and a mean age of 77 years.

Table 2.1 Human Tissue Description

							αSyn pathology		
Case#	**Diagnosis**	**Age**	**Sex**	**PMI**	**Braak**	**Plaques**	**Cortex**	**Amygdala**	**Midbrain**
1720	Healthy	73	Male	5.25	2	1			
1731	Healthy	74	Female	7.5	2	1			
1761	Healthy	86	Male	<24	1	0			
1052	Healthy	87	Male	8	2	1			
1728	Healthy	>89	Female	8	4	0			
198	DLB	72	Male	<24	4	3	yes	yes	yes
727	DLB	76	Female	19	6	3	yes	yes	yes
887	DLB	67	Female	12	5	3	yes	yes	yes
1764	DLB/PD	73	Male	33	2	1	yes	yes	yes
1783	Hippocampal sclerosis/DLB	78	Male	48	2	1	yes	yes	yes

1850	DLB/PD	85	Male	31.5	1	1	yes	yes	yes
1859	DLB/PD	66	Male	3.5	2	0	yes	yes	yes

ICV injection

Using an IACUC-approved protocol, intracerebroventricular (ICV) injections were performed using a modified free-hand method as previously described (Clark, Vivonia et al. 1968; Dineley, Kayed et al. 2010). Briefly, mice were deeply anesthetized with an intraperitoneal injection of ketamine/xylazine mixture (65 and 7.5 mg/kg, respectively), the scalp was shaved and an incision was made through the midline to expose the top of the skull. A 28-gauge needle held with a forceps was lowered 1 mm posterior and 1 mm lateral of the bregma. The needle was connected with 0.28 mm polyethylene tubing to a 25-µl syringe driven by an electronic programmable micro-infuser (Harvard Apparatus, Holliston, MA), which was used to deliver 3 µl/mouse at a rate of 6 µl/min. After injection, the needle was left in place for 1 min and the surgical wound stitched before allowing the mouse to recover while lying on a heated pad under a warm light. Reliability and consistency of injections was routinely tested during actual experiments by injecting India ink in parallel animals and macroscopically observing proper coloration of the ventricles (Dineley, Kayed et al. 2010). 18 h after ICV, animals were given an IP injection of either 1% DMSO in 0.9% saline or CaN inhibitor FK506 diluted in 0.9% saline at 10 mg/kg (LC Laboratories, Woburn, MA).

Immunohistochemistry

Five mm sections of human frontal cortex were taken out of storage at -80°C and equilibrated to -20°C before embedding in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA). 10 μm frontal cortex sections were cut and affixed to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), for storage at -80°C until use. The slides were then rinsed in 0.1M PBS and fixed in ice-cold 4% paraformaldehyde for 15 min in RT. After two washes in 0.1M PBS, the sections were blocked and permeabilized for 1 h in 0.1M PBS containing 10% goat serum, 0.03% Triton-X, and 0.1% phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA). Incubation with primary antibodies in 0.1M PBS containing 10% serum and 0.1% phosphatase inhibitor was carried out overnight at 4°C. Following two PBS washes, the slides were incubated for 1 h in RT with Alexa Fluor 488 or 594 secondary antibodies (1:600; Invitrogen, Carlsbad, CA) in 0.1M PBS containing 10% serum and 0.1% phosphatase inhibitor. Slides were again rinsed twice in PBS and once in distilled water. Human slides were incubated for 8 min with 0.3% Sudan Black B (EMD Chemicals, Gibbstown, NJ) in 70% ethanol to block lipofuscin autofluorescence (Romijn et al., 1999). After two more rinses in distilled deionized water, Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was applied (Vector Laboratories, Burlingame, CA), and coverslips were mounted. Nail polish was applied to seal the edges and signal.

Nitration using Tetranitromethane

In the fume hood, 10 µl of 1% TNM in ethanol is added to 100 µl of 1 mg/ml αSyn and stirred for ten minutes. Another 10 µl of the TNM solution is added and stirred again for ten minutes. Then, 30 µl of 8 M urea is added to quench the reaction. Finally, the

reaction is then dialyzed in 20% ethanol in TBS and the solution is exchanged four times to eliminate unreactive TNM.

Nuclear fractionation

Tissue was homogenized and fractionated using a commercially available nuclear/cytosol fractionation kit (BioVision Incorporated, Mountain View, CA, Catalog# K266) according to the manufacturer's instructions.

Phosphatase activity assay

The activity of CaN (PP2B) and combined activity of PP1/PP2A were assayed from lysed cytosolic cell and tissue extracts using a commercially available colorimetric kit (EMD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Preparation of αSyn oligomers and fibrils

The formation of αSyn aggregates (oligomers and fibrils) was achieved via seeding monomeric αSyn with a fractional small amount (1:140 v:v) of amyloid beta (Aβ) oligomers (oligomerization described below). The final concentration of contaminant Aβ oligomers in the αSyn solution used in these experiments was 20 to 250 times lower than the minimum Aβ effective concentration. Seed Aβ oligomers were prepared by dissolving 0.3 mg of lyophilized Aβ42 (W.M. Keck Facility, University of Yale) in 200 µl of hexafluoro-2-propanol (Sigma-Aldrich) for 10–20 minutes at room temperature. The resulting Aβ solution was added to DD H_2O in a siliconized Eppendorf tube to a final concentration of 66 µM. A magnetic stir bar was placed in the tube and holes were

placed in the cap to promote evaporation of the HFIP, and the solution was stirred for 48 hours. For preparation of αSyn oligomers of fibrils, 0.1 mg of lyophilized powder of αSyn (rPeptide) was dissolved in 230µl H2O and seeded with the Aβ oligomers (1:140 v:v) and stirred 20 min for oligomers and 5 days for fibrils. The resulting αSyn aggregates were tested using Western blot for quality control purposes.

Table 2.2 Primary antibodies

Antigen	**Antibody and Vendor**	**Host Species**	**Dilution**
αSyn	4D6; Signet Laboratories Inc.	Mouse	WB -1:5000 IHC – 1:800
αSyn	C-20; Santa Cruz Biotechnology	Rabbit	WB - 1:500
Phospho-CREB (Ser 133)	Anti-pCREB; Millipore	Rabbit	WB - 1:1000 IHC – 1:100
CREB	86B10; Cell Signaling	Mouse	WB - 1:1000 IHC – 1:100
Calcineurin A	610259; BD Biosciences	Mouse	WB - 1:1000
Nitro-synuclein	NSyn; Upstate	Mouse	WB - 1:5000
Nitro-Tyrosine	NTyr; Upstate	Rabbit	WB - 1:10000
Beta Actin	Bactin; Sigma	Mouse	WB - 1:10000
NeuN	NeuN; Cell Signaling	Mouse	WB – 1:1000 IHC – 1:100

SEAP transfection

Transfections were performed using liposome-mediated plasmid introduction in SY5Y cells. The liposome used was DMRIIE-C (Invitrogen, Carlsbad, CA). Cultures at 40% to 50% confluence received 1.2 pmole mL-1 of DNA coupled to DMRIIE-C at a ratio of 1:3, diluted in serum-free OptiMEM medium (Invitrogen, Carlsbad, CA). Three hours after transfection, the liposome-DNA mix was replaced with fresh culture medium with serum and the cells were allowed at least 48 h of recovery prior to addition of αSyn with or without 10 μM forskolin (Sigma, St. Louis, MO). Secreted alkaline phosphatase activity (SEAP; Clontech, Palo Alto, CA) was used as a direct measure of the effect of test compounds on transcription factor activity. Plasmids containing the SEAP gene coupled to either a control promoter (pTAL-SEAP) or enhancer sequences specific to binding CRE (CRE-SEAP) were transiently transfected as described previously. SEAP activity was assayed directly from the culture medium using the Great EscAPe Chemiluminescent Detection Kit according to manufacturer's instruction (Clontech, Palo Alto, CA).

Seeding αSyn with NαSyn

200 μl of water is added to a 0.1 mg aliquot of lyophilized αSyn, and the solution is split equally into two different eppendorph tubes. In one of the tubes, 10 μl of nitrated αSyn monomer (500 μg/ml) is added and nothing is added to the other tube (this sample will be control fibrils). Both samples are then stirred for five days.

Urea/Proteinase K treatment

100 μg of 1 mg/ml solution of brain homogenate were treated with 100 μg/ml proteinase K (PK, Sigma Aldrich #P2308) for 60 minutes at 37°C, whereas a parallel set

of samples were treated with 8 M urea (Sigma Aldrich #U-1250) in boiling water for 20 minutes. The reactions were stopped by adding SDS sample buffer and boiling for 10 minutes. The samples were then resolved on SDS polyacrylamide gels, transferred to nitrocellulose membrane and probed with a rabbit polyclonal anti-αSyn antibody (C-20, Santa Cruz cat# sc-7011-R).

Western blotting

Tissue was homogenized either into nuclear fractions or in lysis buffer containing 50 mM Tris HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM PMSF, and 1% protease cocktail inhibitors (Sigma, St. Louis, MO). Following homogenizing, the tissue was centrifuged at 20,000 g for 5 min, and the supernatants collected. Protein samples containing 100 µg of proteins as determined by the BCA assay (Pierce, Rockford, IL), were subjected to SDS-polyacrylamide gel electrophoresis using 10% gels, followed by electrophoretic transfer to nitrocellulose membranes (Biorad, Hercules, CA). Membranes were then incubated overnight at 4^{o}C with the primary antibody, followed by an appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Biorad) against rabbit IgG (for polyclonal primaries) or mouse IgG (for monoclonal primaries). Detection was achieved using Enhanced Chemiluminescence (ECL) (Pierce, Rockford, IL).

Chapter 3: Extracellular alpha synuclein oligomers oppose long term potentiation and impair memory through a mechanism involving calcineurin.

Abstract

Intracellular deposition of fibrillar aggregates of αSyn is a pathological hallmark of such neurodegenerative diseases as PD and DLB, which are clinically characterized by motor dysfunction and cognitive decline. However, recent evidence indicates that small αSyn oligomeric aggregates which precede fibril formation may be the most neurotoxic species and can be found extracellularly where they may be key to inter-neuronal transmission of the disease neuropathology. This new evidence has changed the view of pathological αSyn aggregation from a self-contained cellular phenomenon to an extracellular event and prompted investigation of the putative neurodysfunctional effects of extracellular αSyn oligomers. Here, we examined how oligomeric αSyn affects neuronal function and memory when applied extracellularly. We found that oligomeric αSyn, but not monomeric or fibrillar αSyn, increased intracellular Ca2+ levels, induced the activity of calcineurin (CaN), and decreased the activity of CREB in human SY5Y neuroblastoma cells. Similar induction of CaN and inhibition of active pCREB were observed when αSyn oligomers, but not monomers or fibrils, were applied to organotypic brain slices. As well, αSyn oligomers induced CaN and decreased pCREB when acutely injected into the lateral ventricles of wild-type mice. Notably, all these events could be reversed by inhibition of CaN with FK506. These results indicate that extracellular αSyn oligomers can impact neuronal function and produce memory deficits through mechanisms that invoke CaN activity.

Preface

The following experiments were a collective effort done in my lab except Figure 3.4B, which was done independently by me.

Introduction

The signature event in synucleinopathies such as Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), is the misfolding, aggregation, and intracellular accumulation of alpha synuclein (αSyn) (Goedert 2001). αSyn is a 140 amino acid long protein that remains relatively unfolded under non-disease conditions and is abundant in pre-synaptic terminals of neurons where it may play a role in vesicular trafficking (Burre, Sharma et al. 2010). αSyn is prone to misfolding and aggregating into fibrils that are the main components of Lewy bodies (LB) and Lewy neurites (Braak et al., 1999 (Spillantini, Crowther et al. 1998; Braak, Sandmann-Keil et al. 1999). These deposits are accompanied by progressive neuronal dysfunction and eventually death of affected neuronal populations (Braak and Del Tredici 2008). Behavioral and cognitive deficits usually accompany these pathological changes (Braak, Rub et al. 2005; Caviness, Lue et al. 2011). The discovery of three missense mutations in the SNCA gene (encoding αSyn) that are linked to rare cases of familial PD (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998; Zarranz, Alegre et al. 2004), have highlighted the potential importance of αSyn in the pathogenesis of these diseases, likely due to toxic gain-of-function. Indeed, αSyn knock-out mice are normal, but mice overexpressing αSyn display synucleopathic phenotype (Kahle 2008). However, αSyn fibrils that comprise LBs are thought to be protective on account of their possible sequestration activity of more toxic oligomeric forms of αSyn; oligomeric αSyn is considered an intermediate in the aggregation pathway (Olanow, Perl et al. 2004). αSyn oligomer toxicity is exemplified by a juvenile form of PD, where extensive neurodegeneration occurs even in the absence of LB formation (Takahashi, Ohama et al. 1994; Mori, Kondo et al. 1998; Olanow, Perl et al. 2004). Also, microscopic and biochemical analysis showed that LB-bearing neurons do not exhibit cytotoxicity

(Gertz, Siegers et al. 1994; Tompkins and Hill 1997). More directly, *in vitro* and *in vivo* experiments recently illustrated that αSyn oligomers are toxic to neural cells whereas fibrils are not (Kayed, Head et al. 2003; Putcha, Danzer et al. 2010; Winner, Jappelli et al. 2011). Classically, the aggregation and deposition of αSyn has been considered an intracellular phenomenon (McNaught and Olanow 2006). However, more recent evidence demonstrates the existence of extracellular αSyn oligomers and suggests that they play key roles in disease progression (Lee 2008; Brown 2010). For example, αSyn oligomers are released from cultured cells (Emmanouilidou, Melachroinou et al. 2010), are detectable in the spinal fluid and plasma of PD patients (Borghi, Marchese et al. 2000; El-Agnaf, Salem et al. 2003; El-Agnaf, Salem et al. 2006) as well as in the soluble protein fraction from brains of DLB patients (Paleologou, Kragh et al. 2009). In addition, αSyn can be transmitted from neuron to neuron (Desplats, Lee et al. 2009) or neuron to astroglia (Lee, Suk et al. 2010); fetal tissue grafts in the brain of PD patients acquire LB pathology (Li, Englund et al. 2008; Chu and Kordower 2009); and stem cells or fetal tissue transplanted into the CNS of Tg mice overexpressing human αSyn show intracellular deposits formed by host αSyn (Desplats, Lee et al. 2009; Hansen, Angot et al. 2011). Collectively, these findings suggest the presence of extracellularly released αSyn oligomers The presence of extracellularly-released αSyn oligomers suggests that they play a central role in synucleinopathic human diseases (Lee 2008). It also illustrates the importance of determining the effects of extracellular αSyn on neurons, and specifically at synapses where αSyn is thought to be released from the αSyn-enriched pre-synaptic terminals (Schulz-Schaeffer 2010). With this goal in mind, the present study used *in vitro*, *ex vivo* and *in vivo* models to investigate the effects of extracellularly-applied αSyn oligomers on Ca2+-related signaling, hippocampal synaptic plasticity and cognitive function. We focused on two key plasticity, learning and memory proteins: calcineurin (CaN), a CNS-abundant Ca2+/Calmodulin-dependent phosphatase (Mansuy,

Winder et al. 1998; Mansuy 2003), and cAMP response element binding protein (CREB), a transcription factor indirectly regulated by CaN (Bito, Deisseroth et al. 1996; Pittenger and Kandel 1998; Silva, Kogan et al. 1998; Kinney, Sanchez-Alavez et al. 2009).

Results

The Ca^{2+}/calmodulin-dependent phosphatase calcineurin (CaN) is a key negative modulator of synaptic plasticity and memory function (Mansuy 2003; Lee, Jee et al. 2004). We have previously reported that CaN hyperactivation mediates some of the detrimental effects that oligomers of amyloid-beta (Aβ) exert on synapses and cognition in Alzheimer's disease models (Dineley, Hogan et al. 2007; Reese, Zhang et al. 2008; Dineley, Kayed et al. 2010). These neurotoxic aggregate species share structural and functional similarities with αSyn oligomers (Glabe and Kayed 2006). As such, we chose to examine whether specific aggregates of αSyn also induce the activity of the phosphatase CaN and subsequent detrimental effects on synaptic and cognitive function through inactivation of CREB. We employed monomeric, oligomeric, and fibrillar conformations of αSyn prepared as previously described (Kayed, Head et al. 2003).

Consistent with previous reports on other oligomeric amyloid proteins (Demuro, Mina et al. 2005), treatment of human neuroblastoma SY5Y cells with oligomeric αSyn induced an increase in intracellular Ca^{2+} levels (Fig. 3.1); monomeric and fibrillar αSyn, even though added to the very same cells that were responsive to oligomeric αSyn, were ineffective.

Increased intracellular Ca^{2+} may result in activation of the Ca^{2+}/calmodulin-dependent CaN. Therefore, we next determined phosphatase activity and cell survival in SY5Y cells, organotypic brain slices, and C57 wildtype mice treated with monomeric, oligomeric, and fibrillar αSyn (Figs. 3.2a, 3.2b, and 3.2c). We observed that only oligomeric αSyn increased the activity of CaN. Moreover, in the wildtype mice the

hippocampus (HIPP), amygdala (AMYG), medial cortex (MCTX), anterior cortex (ACTX), basal forebrain/septum (BFA), and cerebellum (CB) were assessed for CaN activity on freshly homogenized tissue (Fig. 3.2c). We found that CaN activity was significantly elevated in HIPP, AMYG, MCTX, and BFA of mice subjected to icv injection of αSyn compared to saline-treated mice. FK506 restored CaN activity to control levels in these brain areas of αSyn-treated mice.

One of the proteins dephosphorylated either directly or indirectly by CaN is CREB. Dephosphorylation of CREB deactivates this transcription factor that normally transcribes proteins involved with synaptic plasticity, learning, and memory (Josselyn, Kida et al. 2004; Hotte, Thuault et al. 2007). In order to ascertain if these events may be triggered by αSyn oligomers, we first sought to determine if the oligomeric and fibrillar forms of αSyn had any effect on the activity of CREB (Fig 3.3). SY5Y cells were transiently transfected with a cDNA construct encoding a secreted alkaline phosphatase (SEAP) reporter gene driven by a CREB-sensitive promoter (pCRE-SEAP).

All cells were additionally transfected with a vector encoding renilla luciferase to ensure equal transfection efficiency among samples (data not shown). Twenty-four hours after transfection, cells were treated with 2 μM of either oligomeric or fibrillar αSyn, in the presence or absence of the adenylate cyclase (AC) activator forskolin (10 μM). The amount of SEAP in the culture medium, assayed three hrs after treatment – a time point well before the observance of any cell death, is indicative of CREB-driven transcription. Under basal conditions, release of SEAP was not significantly diminished by treatment with oligomeric αSyn although a trend was apparent.

Cells treated with fibrillar αSyn exhibited no such reduction in SEAP. Since the level of CREB driven transcription in SY5Y cells is quite low under basal conditions, we performed a second experiment using cells treated with forskolin. This drug greatly induced PKA-promoted CREB transcription activity, evidenced by the increased SEAP

release (Fig. 3.3 right). Under these conditions, treatment of the stimulated cells with oligomeric αSyn significantly opposed the release of SEAP; those treated with fibrillar αSyn were not affected.

Next, we expanded on the initial findings from SY5Y cells showing decreased CREB-induced transcription to *ex vivo* rat brain slices and wildtype mice to investigate pCREB levels after treatment with αSyn oligomers. Figure 3.4a shows an αSyn time course experiment performed in acutely cultured rat brain slices to assess pCREB by Western blot. pCREB was maximally decreased in total protein extracts from brain slices treated with oligomeric αSYn for 15 minutes; pCREB recovered by the 30 to 60 minute time points. Conversely, monomeric and fibrillar αSyn did not affect the phosphorylation of CREB. In fact, treatment with monomer appeared to have the opposite effect (although it did not reach statistical significance)**.** The effect on pCREB by oligomeric αSyn closely mirrored the dramatic rise in CaN activity seen in Fig. 3.2 which also peaked at 15 minutes and remained significantly increased up to 60 minutes (Fig. 3.4a). Immunohistochemical examination of the hippocampus of mice injected icv with either saline or αSyn oligomers (Fig. 3.4b) confirmed that neuronal pCREB was significantly decreased by αSyn oligomers. As well, expression of total CREB remained unchanged after icv injection of αSyn oligomers, although a trend toward reduced expression was noticeable.

FIGURES

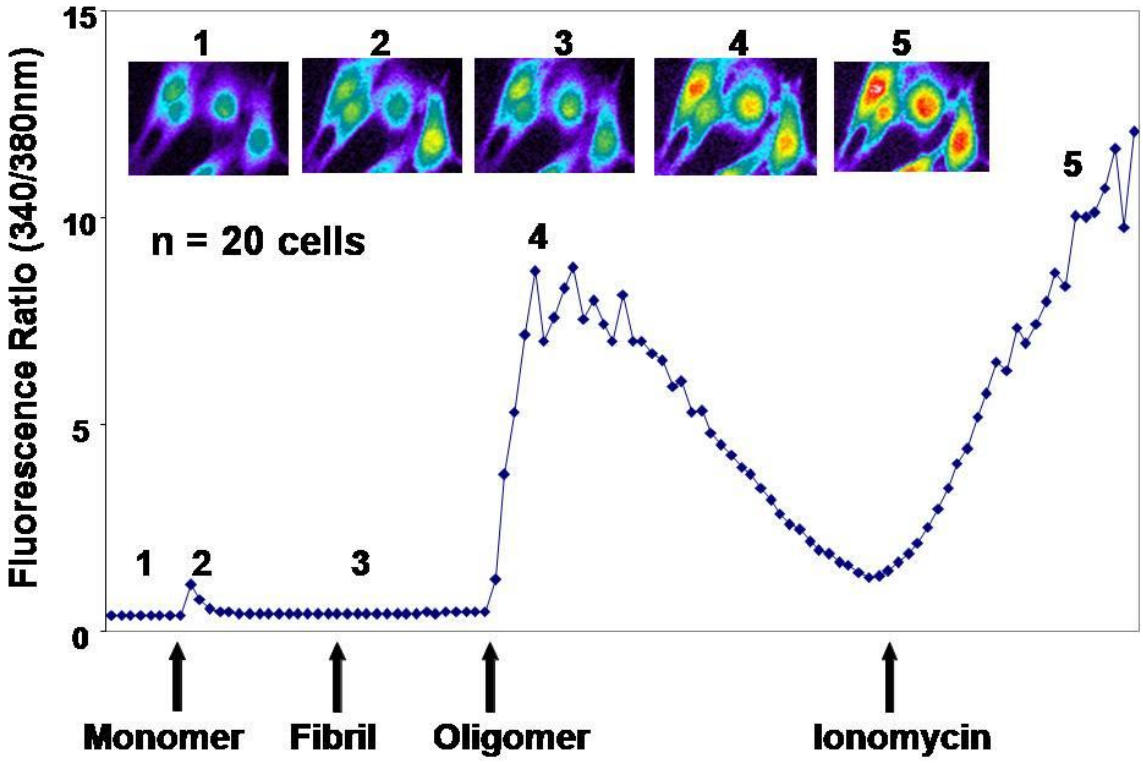
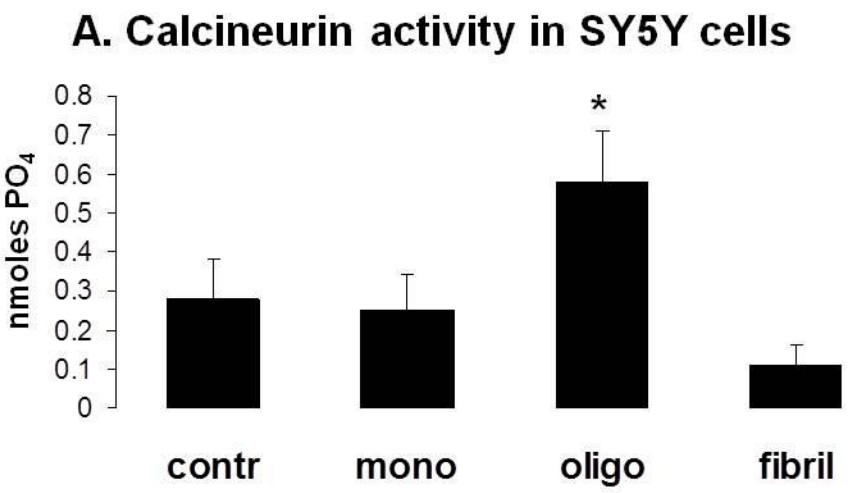
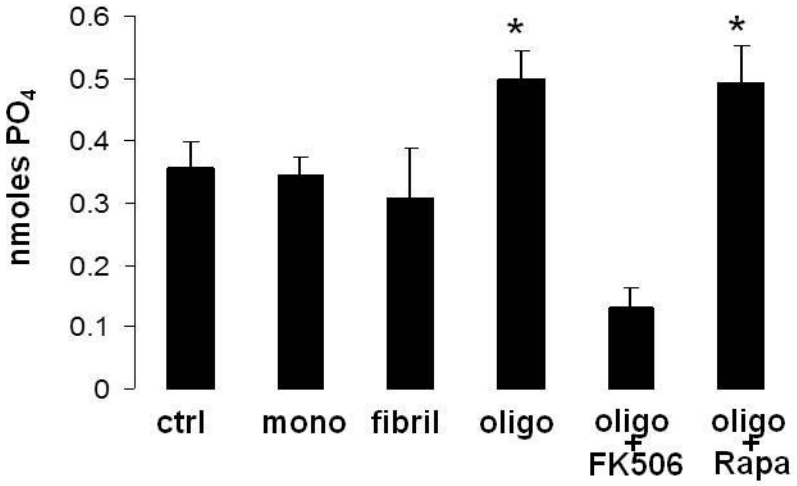


Figure 3.1 αSyn oligomers, but not monomers or fibrils, increases intracellular Ca^{2+} levels in SY5Y cells. Graph showing a 35-minute time course of Ca^{2+} dependent fluorescence recorded and averaged from 20 fluo-3 loaded SY5Y human neuroblastoma cells in response to consecutive application of αSyn monomers, fibrils, and oligomers (4μM for 10 min each). Cells were challenged with ionomycin (4μM) at the conclusion of the experiment. Each point represents the average of the 340/380 nm readings from 20 individual cells. Representative images on top depict the response of 4 individual cells at the time points indicated by the corresponding number on graph. Warmer colors correspond to a higher level of fluorescence.


A. Calcineurin activity in SY5Y cells
nmoles PO4
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
contr
mono
oligo
fibril


B. Calcineurin activity in rat brain slices
nmoles PO4
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
ctrl
mono
fibril
oligo
oligo + FK506
oligo + Rapa

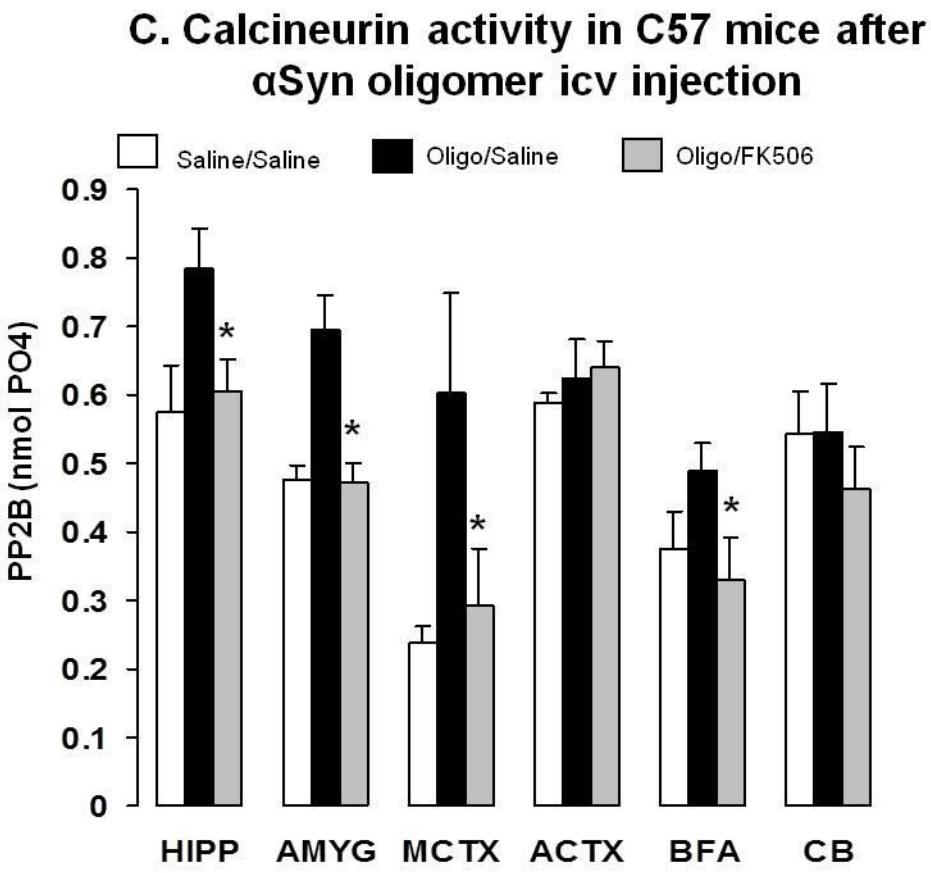


Figure 3.2 Selective induction of CaN activity and CaN-dependent cell death by oligomeric αSyn in SY5Y cells.

A. CaN (top) and combined PP1+PP2A (bottom) activity in human SY5Y neuroblastoma cells treated for 24 hr with 2 μM αSyn monomers, oligomers or fibrils. Graph is representative of two independent experiments returning similar results. N=3 independent measurements per condition; *: $p<0.05$ vs. control group (ANOVA).

B. Calcineurin (top) and d PP1+PP2A combined activity (bottom) assayed in the same brain slices shown in A at the end of the LTP recording. Columns represents means ± S.D.; N=3 per group; *: $p<0.05$ vs. control (ANOVA).

C. Calcineurin (top) and PP1+PP2A combined activity (bottom) in the hippocampus (HIPP), amygdala (AMYG), medial cortex (MCTX), anterior cortex (ACTX), basal

forebrain area (BFA) and cerebellum (CB) of the same mice sacrificed at the end of the FC memory tests.

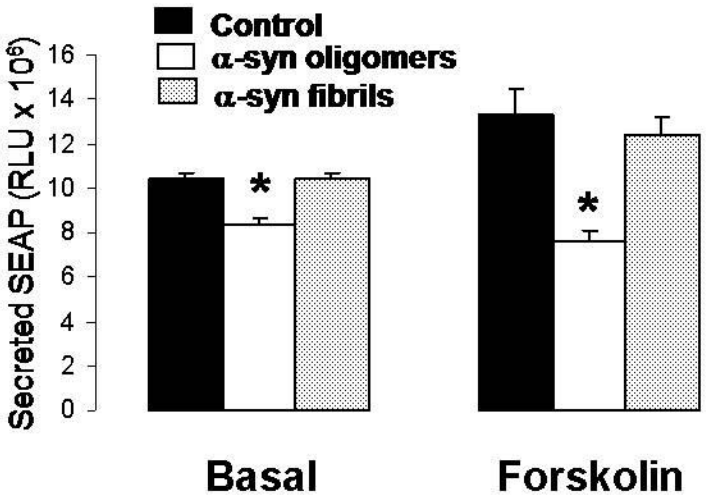
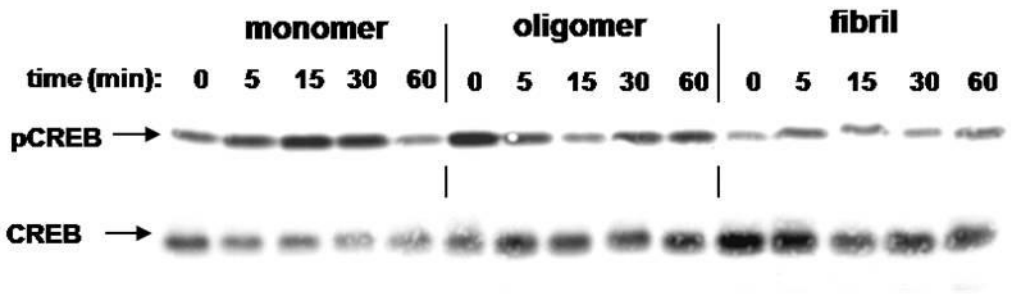
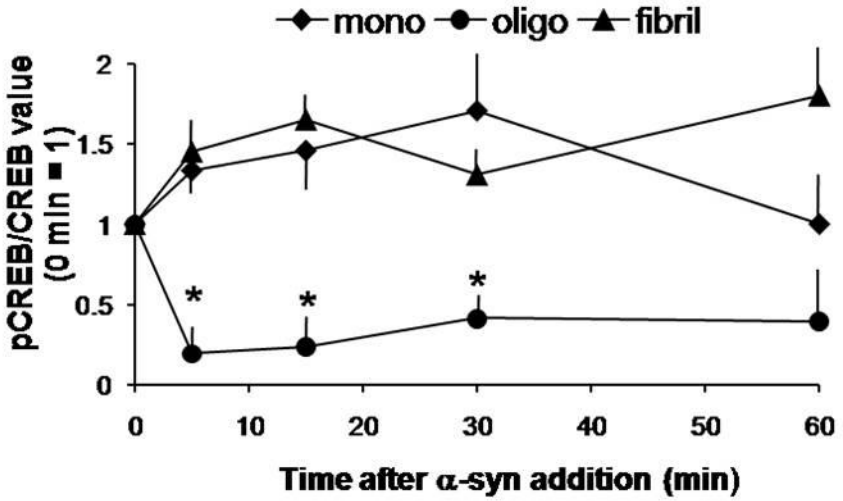


Figure 3.3 CREB-promoted transcriptional activity is inhibited by oligomeric αSyn in both basal and AC/PKA-stimulated conditions in SY5Y cells. Release of the reported gene product SEAP in SY5Y cells transiently transfected with the CREB-sensitive CRE-SEAP construct and treated for 3 hr with oligomeric or fibrillary αSyn (2 μM) in the presence or absence of the AC/PKA activator forskolin (10 μM). N=6 replicates per group; *: $p<0.01$ vs. control cells (ANOVA).

A


monomer
oligomer
fibril
time (min): 0 5 15 30 60 0 5 15 30 60 0 5 15 30 60
pCREB
CREB


mono
oligo
fibril
pCREB/CREB value (0 min = 1)
2
1.5
1
0.5
0
*
*
*
0
10
20
30
40
50
60
Time after α-syn addition (min)

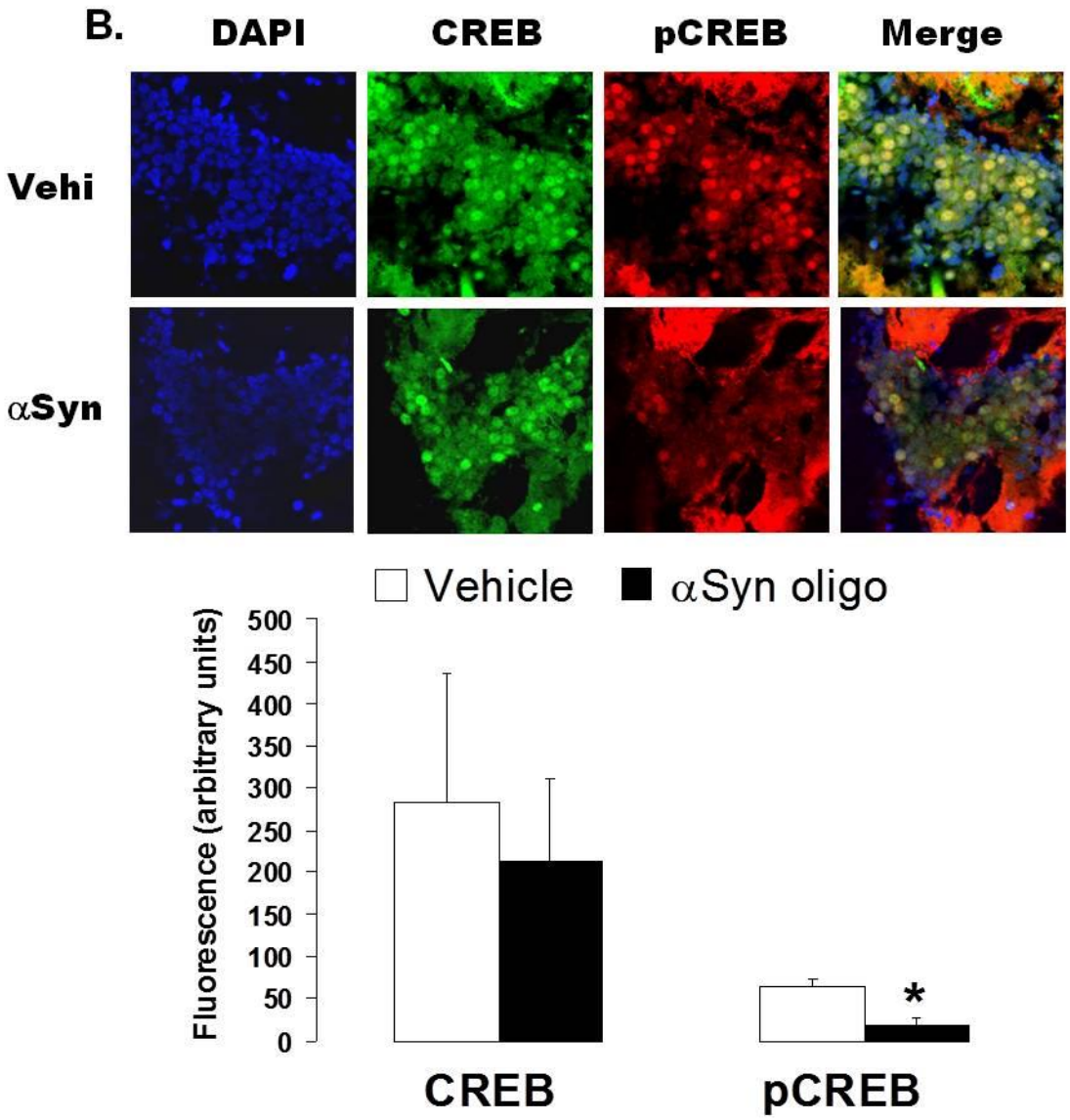


Figure 3.4 Oligomeric αSyn reduces pCREB levels in organotypic rat brain slices and wildtype mice after treatment with αSyn oligomers.

A. Representative Western blot (top) detecting pCREB in total protein extracts from organotypic brain slices treated with 0.75 µM of monomeric, oligomeric and fibrillar αSyn for the time length shown. The blot was stripped and re-probed for total CREB to control for sample gel loading. Densitometry band quantification (bottom) confirmed that pCREB levels were significantly reduced by αSyn oligomers but not monomers or fibrils. Graph

represent average from 3 independent experiments. *: $p<0.05$ vs. time 0 control (ANOVA).

B. Representative confocal images illustrating pCREB expression (in red) in the granule layer of the dentate gyrus in the hippocampus of mice injected icv with vehicle or αSyn oligomers (100 nmoles/3 ml/mouse) 24 hr earlier. Time lapse between icv injection and sacrifice corresponded to the time lapse between icv injection and FC training as illustrated in (A). Sections were further co-stained for total CREB (in green) and mounted using a DAPI-containing mounting medium to visualize cell nuclei (in blue). The bar graph at the bottom illustrates the results from the image analysis of captured confocal images of sections co-stained for pCREB and CREB. N=3 each vehicle- or αSyn oliomer-injected mice; *: $p<0.01$ vs. control (ANOVA).

Discussion

Taken together, our results indicate that small oligomeric aggregates of αSyn applied extracellularly to *in vitro*, *ex vivo* and *in vivo* experimental models exert profound effects on neuronal signaling involved with synaptic plasticity and memory deficits. Across the board, we found these effects to be specifically attributable to αSyn oligomeric preparations rather than monomeric or fibrillar concoctions. A model emerges whereby extracellularly applied oligomeric αSyn induces increased intracellular Ca^{2+}, CaN activation, and the dephosphorylation of a key signal transduction molecule required for synaptic plasticity, learning and memory. It is therefore prudent to propose that extracellular αSyn oligomers, through a role compromising signal transduction mechanisms that underlie synaptic function, may play a role in cognitive decline that accompanies certain synucleopathies such as PD and DLB (Schulz-Schaeffer 2010).

We found that treatment of human neuroblastoma SY5Y cells with αSyn oligomers, but not with αSyn monomers or fibrils, quickly elicited an elevation of free intracellular Ca^{2+}. Along with increased intracellular Ca^{2+}, αSyn oligomers caused an increase in the activation of the Ca^{2+}/calmodulin-responsive phosphatase CaN. This increase in CaN activation was also seen on organotypic brain slices and wildtype mice after treatment with αSyn oligomers, demonstrating the same phenomenon of αSyn oligomer-induced hyperactivation of CaN *in vitro*, *ex vivo*, and *in vivo*.

Treatment of SY5Y cells with αSyn oligomers also led to a decrease in CREB-driven transcriptional activity under both basal and forskolin-stimulated conditions; whereas fibrillar αSyn was ineffective. We also observed decreased levels of Ser133 phosphorylated CREB in organotypic brain slices and wildtype mice (without any change in total) that indicate that the transcriptionally competent form of CREB (Bito, Deisseroth et al. 1996) is diminished after treating brain slices with αSyn oligomers or injecting them icv in mice. Decreased pCREB levels were associated with increased CaN activity in brain slices and mice icv-treated with αSyn oligomers. These results suggest that αSyn oligomer-induced activation of CaN results in pCREB dephosphorylation.

As such, we propose that at least one functional outcome of extracellular αSyn oligomers on neuronal networks (e.g., hippocampus) is CaN-dependent deficits in synaptic plasticity and cognitive function. Past LTP studies from Neugebauer's lab show that expression of hippocampal LTP in brain slices is opposed by treatment with αSyn oligomers but not monomers or fibrils. This effect is accompanied by increased CaN activity and decreased CREB phosphorylation (Ser133) and can be reversed by pharmacological inhibition of CaN with FK506, suggesting that CaN is central to this αSyn-mediated impairment of synaptic plasticity. As previously noted, the crucial role of proper CaN activity and pCREB levels in LTP expression has been well established, and

there is ample consensus that LTP constitutes the cellular basis of hippocampus-dependent memory formation.

Indeed, when we injected mice icv with αSyn oligomers, we observed an increase in CaN activity and a decrease in pCREB levels in several CNS brain regions, including hippocampus. Fear conditioning studies done in Dineley's lab showed icv injection of αSyn oligomers was accompanied by a significant contextual fear conditioning impairment in this hippocampus-dependent memory task. Such αSyn oligomer-induced memory deficit was completely abolished by treatment of the mice with FK506, which also returned CNS CaN activity and pCREB levels to normal values. It is important to note that in both LTP and behavior experiments, FK506 was applied minutes to hours after αSyn oligomers, respectively, indicating that the impairments in LTP or memory elicited by αSyn oligomers under these experimental conditions are reversible and further suggests that these effects of αSyn oligomers are not the result of αSyn-induced cell death.

Indeed, we detected no change in the level of phosphorylated BAD assayed in brain tissue samples from the same animals receiving icv injection of αSyn oligomers and behavioral analysis (data not shown). Although CaN-mediated dephosphorylation/activation of the pro-apoptotic protein BAD has been described as a central event in neuronal apoptosis induced by a variety of toxic stimuli, including increased intracellular Ca^{2+} levels (Wang, Pathan et al. 1999; Springer, Azbill et al. 2000; Shou, Li et al. 2004; Yang, Omori et al. 2004), unchanged levels of BAD dephosphorylation in the brain of mice icv-injected with αSyn oligomers indicates that the observed memory deficit does not appear to involve overt neuronal death.

The presence of extracellular misfolded αSyn oligomers in neurodegenerative disorders has recently gained much attention because of its potential, albeit yet unclear role in disease progression (Lee 2008; Schulz-Schaeffer 2010). Recent investigations

have implicated that αSyn oligomers are released via an atypical secretory pathway. For example, αSyn oligomers are released in the culture medium of cells overexpressing human αSyn (Emmanouilidou, Melachroinou et al. 2010) and αSyn oligomers have been found in the spinal fluid and plasma of PD patients (El-Agnaf, Salem et al. 2003; El-Agnaf, Salem et al. 2006). Like all neurodegenerative diseases, PD has a typical neuroanatomical progression that affects the brainstem foward to the frontal cortex (Braak, Del Tredici et al. 2003), thus suggesting a prion-like mechanism of inter-cellular transmission of misfolded αSyn species (Angot, Steiner et al. 2010; Brundin, Melki et al. 2010). Indeed, neuron-to-neuron and neuron-to-astroglia transmission of misfolded αSyn species has been demonstrated *in vitro* and *in vivo* (Desplats, Lee et al. 2009; Lee, Suk et al. 2010). Furthermore, αSyn pathology deriving from the host αSyn pool has been found in fetal tissue grafted into the CNS of PD patients (Li, Englund et al. 2008; Chu and Kordower 2009) as well as in dopaminergic neurons grafted in the CNS of αSyn transgenic mice (Hansen, Angot et al. 2011). This collectively implies that aggregated αSyn species may gain access to the extracellular space where they contact and are taken up by neighboring neurons. This mechanism may be of particular significance at the synapse, where αSyn oligomers can be released presynaptically and may impact post-synaptic elements. Indeed, abundant αSyn aggregates are found at presynaptic terminals in PD and DLB (Schulz-Schaeffer 2010) and their pathological presence was associated with loss of pre- and post-synaptic markers (Kramer and Schulz-Schaeffer 2007) as well as retraction of dendritic spines in affected areas (Zaja-Milatovic, Milatovic et al. 2005; Zaja-Milatovic, Keene et al. 2006; Revuelta, Rosso et al. 2008). This neuroanatomical evidence suggests a significant loss of synapses occurring in the brains of PD and DLB patients that may underscore the cognitive symptoms of these diseases; it also introduces a neuron loss-of-function component to the pathology of

diseases like PD whose overall clinical manifestations have classically been ascribed to overt neuronal death in specific brain regions.

Therefore, our current results reveal a previously undocumented mechanism whereby small oligomeric αSyn species that have gained extracellular localization may impact healthy neurons and disturb synaptic plasticity, thus impacting memory function and cognitive ability. Our results further suggest that such pathological mechanisms may be effectively targeted by pharmacological inhibition of CaN and possibly by future immunotherapies aimed at scavenging extracellular oligomeric αSyn.

Chapter 4: Soluble alpha synuclein oligomers are associated with reduced pCREB and increased calcineurin in frontal cortex from patients with dementia with Lewy bodies

Abstract

Dementia with Lewy Bodies (DLB) is a neurodegenerative disorder that, similar to Parkinson disease, is characterized by the misfolding, aggregation and deposition of the protein alpha synuclein (αSyn). Recent evidence indicates that rather than the larger fibrils found in Lewy body deposits, the most toxic αSyn species in the aggregation pathway are the intermediate oligomeric species. However, the mechanism of action of αSyn oligomers remains elusive. Our previous studies have shown that proteins involved in synaptic welfare and memory function such as the protein phosphatase calcineurin (CaN) and pCREB (a transcription factor negatively regulated by CaN and important for synaptic integrity and memory) mediate neurotoxicity of αSyn oligomers *in vitro, ex vivo,* and *in vivo* and results in deficits in synaptic plasticity and memory function. On this

basis, I tested *the hypothesis that similar effects on synaptic signaling can be observed in human brains from DLB patients associated with specific αSyn oligomeric species*. Here I report that in the brains of DLB patients there is an increase in truncated CaN, a significant reduction of active pCREB, and this is associated with the appearance of αSyn dimers. Overall our results support the idea that increased CaN activity (as reflected here by CaN truncation and perturbed levels of the downstream target pCREB) mediates some of the neuro-dysfunctional effects of aggregated αSyn in humans and may thus constitute a viable pharmacological target.

Introduction

Dementia with Lewy Bodies (DLB), similar to Parkinson Disease (PD), is a debilitating and progressive neurodegenerative disease pathologically marked by the misfolding, aggregation and accumulation of the toxic protein alpha-synuclein (αSyn). αSyn is a naturally unfolded 14kDa protein primarily located at the pre-synapse of neurons and in glia (Maroteaux, Campanelli et al. 1988). Even though αSyn's normal function remains unclear, studies suggest it to be involved in regulation of vesicle trafficking (Jenco, Rawlingson et al. 1998). However, inactivation of the αSyn gene using homologous recombination does not lead to neurological dysfunction, suggesting that αSyn loss-of-function most likely does not play a role in neurodegeneration (Abeliovich, Schmitz et al. 2000). Rather, a gain-of-function of the toxic misfolding and aggregation of αSyn is a more likely etiological component in disease phenotype. Further support of αSyn as the correlate in synucleinopathies have been the discoveries of three missense mutations (A53T, A30P, and E46K) in the αSyn protein that are linked to rare forms of familial PD (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998; Zarranz,

Alegre et al. 2004). Moreover, αSyn is the primary protein component of the lesions (Lewy Bodies) in brains of patients with the more common, sporadic PD. However, recent evidence has revealed that the higher molecular weight fibril aggregates found in Lewy Bodies might actually be protective by sequestering the toxic oligomeric intermediates (Olanow, Perl et al. 2004). This complements other studies showing αSyn oligomers are the most toxic species in the aggregation pathway both *in vitro* and *in vivo* (Sharon, Bar-Joseph et al. 2003; Demuro, Mina et al. 2005).

αSyn oligomers are heterogeneous (dimer, trimer, etc.) and are similar in structure to other oligomers formed by amyloid proteins whose presence characterizes other neurodegenerative diseases as demonstrated using conformational antibodies, electron microscopy and atomic force microscopy (Kayed, Head et al. 2003; Kayed, Sokolov et al. 2004; Demuro, Mina et al. 2005). One oligomeric intermediate in the amyloidogenic pathway is represented by protofibrils, which form pore-like structures that can readily integrate into the cell membrane from either intracellular or extracellular sides, thus perturbing membrane stability and consequently increasing intracellular Ca^{2+} levels (Kayed, Head et al. 2003; Demuro, Mina et al. 2005; Glabe and Kayed 2006).

While αSyn is most abundant intracellularly at pre-synaptic terminals, growing evidence suggests that it can also gain access to the extracellular space where αSyn may affect neighboring cells. Evidence that αSyn can bypass cell membranes is provided by the observation of the presence of αSyn in the CSF and plasma of PD patients (Borghi, Marchese et al. 2000; El-Agnaf, Salem et al. 2003) as well as studies showing that αSyn overexpressing neurons release αSyn in the culture medium (Lee, Patel et al. 2005). Recent studies have also shown that fetal tissue grafted into the CNS of PD patients eventually develops αSyn aggregates and Lewy bodies (Kordower, Chu et al. 2008; Li, Englund et al. 2008), suggesting the possibility of inter-neuronal

transmission of extracellular αSyn aggregates (Brundin, Li et al. 2008). Indeed, neuron-to-neuron and neuron-to-astroglia transfer of αSyn species has been reported (Desplats, Lee et al. 2009; Lee, Suk et al. 2010), and αSyn pathology can spread from the host to fetal tissue grafted into the CNS of PD patients (Li, Englund et al. 2008; Chu and Kordower 2009) or to dopaminergic neurons grafted in the CNS of αSyn transgenic mice (Hansen, Angot et al. 2011).

Oligomer-induced rises in intracellular Ca^{2+} profoundly affect neuronal function and eventually cause neuronal death (Volles and Lansbury 2003); consequently, any neuron type exposed to intracellular or extracellular aggregating αSyn (Braak, Del Tredici et al. 2003) can be dysfunctionally affected through permeabilization of the cell membrane, which may be one common event underlying synucleinopathy pathology, and thus represent a target for therapeutic intervention; however, the downstream mechanisms linked to oligomeric αSyn induced neurotoxicity are still poorly understood. Research conducted in our laboratory in the recent past has focused on effects of amyloid oligomers on Ca^{2+} homeostatic regulation and its consequences on the activity of calcineurin (CaN, a Ca^{2+}-sensitive protein phosphatase abundant in the brain and involved in synaptic plasticity and memory function) and concomitant dephosphorylation and deactivation of cAMP response element binding (CREB, a transcription factor involved in synaptic plasticity, learning, and memory). Recently, we have shown that synthetic oligomeric αSyn causes an increase in intracellular Ca^{2+}, CaN activation, and decrease in pCREB *in vitro, ex vivo*, and *in vivo (Neugebauer et al., this issue)*, thus perturbing events that are known to modulate synaptic plasticity and cognition. Indeed, I further showed that αSyn oligomers induce cell death, oppose LTP expression, and impair memory in a CaN-dependent fashion. The present study extends those findings to the actual diseased human brain, demonstrating increased αSyn oligomeric species that

occur concomitant to increased active CaN and decreased pCREB levels in postmortem brain samples from a cohort of DLB patients as compared to age-matched control.

Results

I first examined the presence of αSyn oligomeric species, CaN activity, and pCREB levels in the frontal cortices from DLB patients and age-matched controls. Diagnosis, age, sex, and PMI of the cohort of individuals who donated the cortices employed in this study are shown in Table 4.I. Immunohistochemical analysis revealed the presence of αSyn aggregates in frontal cortices of DLB brains that were not seen in age-matched controls (Fig. 4.1), and western blot analysis demonstrated monomers along with detectable levels of αSyn oligomers (dimers to hexamers, according to the apparent molecular weight) in both DLB and age-matched control samples (Fig. 4.2). Notably, the dimer was increased in the DLB as compared to control cortices while levels of αSyn monomer, trimer, and hexamer did not differ between DLB and control samples. In order to determine the stability of these amyloid aggregates, the human samples were also treated with either urea, which is known to disassemble aggregates through hydrogen bonding interactions, or proteinase K, which is known to digest proteins particularly at aromatic and aliphatic residues. Western blot analysis using a polyclonal anti-αSyn antibody demonstrated high sensitivity to proteinase K both in control and DLB samples and partial sensitivity to urea. The control sample demonstrated a greater sensitivity to urea than DLB while the higher molecular weight hexamers in both control and DLB samples demonstrated greater resistance to urea (Supplementary Fig. 4.1). These results are consistent with a report of αSyn sensitivity to proteinase K and resistance to urea in a Tg A53T mouse model (Tsika, Moysidou et al.).

As suggested in this publication using Tg A53T mice, the urea-resistant bonds seen in the human samples could possibly be caused by posttranslational modifications.

Next, in order to determine activation of CaN in these same samples I measured levels of the 57 KDa, truncated form of subunit A of CaN. Levels of truncated CaN-A reflect levels of the active enzyme in that upon activation of CaN, concurrent truncation of the subunit A occurs to reveal the active site of the enzyme (Liu, Grundke-Iqbal et al. 2005). This indirect approach to measure levels of active CaN in autopsy tissue specimens is necessary since CaN is rapidly deactivated by post-mortem oxidation, thus making direct measurements of CaN enzymatic activity using commercially available kits highly unreliable (Wang, Culotta et al. 1996). Western blot analysis revealed that truncated CaN was increased in the DLB samples relative to age-matched control (Fig. 4.3), suggesting increased CaN activity in the DLB brains. Along with increased truncation of CaN, levels of phosphorylated CREB were dramatically reduced in the nuclear fraction from the `DLB samples as compared to controls, whereas levels of total CREB were relatively unchanged between the two groups (Fig. 4.4). Immunohistochemical analysis of the human brain samples confirmed the marked reduction of pCREB in the samples from DLB patients (Fig. 4.5). Notably, double staining of the sections with NeuN, a neuronal marker, showed that such reduction affected pCREB in both neuronal ($NeuN^+$) and non-neuronal ($NeuN^-$) cells. It is unlikely that the observed changes in αSyn or pCREB levels in these human brains were due to differential protein degradation in samples collected at different post-mortem interval (PMI). Indeed, neither αSyn nor pCREB levels showed any significant correlation with the PMI collection times of the tissues specimens studied here (αSyn 1mer: r^2=0.112, p=0.812; 2mer: r^2=-0.232, p=0.617; 3mer: r^2=0.001, p=0.999; 6mer: r^2=0.222, p=0.633. pCREB: r^2=-0.390, p=0.387). On the other hand, when the levels of pCREB were compared to various αSyn species in individual DLB samples, I found that there was a

significant negative correlation between pCREB and the amount of the αSyn dimer, whereas there was a significant positive correlation between pCREB and the levels of αSyn monomer (Fig. 4.6). The same analysis returned no significant correlations between pCREB and αSyn species in brain samples from age-matched control individuals (1mer: r^2=0.121, p=0.974; 2mer: r^2=0.171, p=0.784; 3mer: r^2=0.009, p=0.989; 6mer: r^2=0.580, p=0.306).

FIGURES

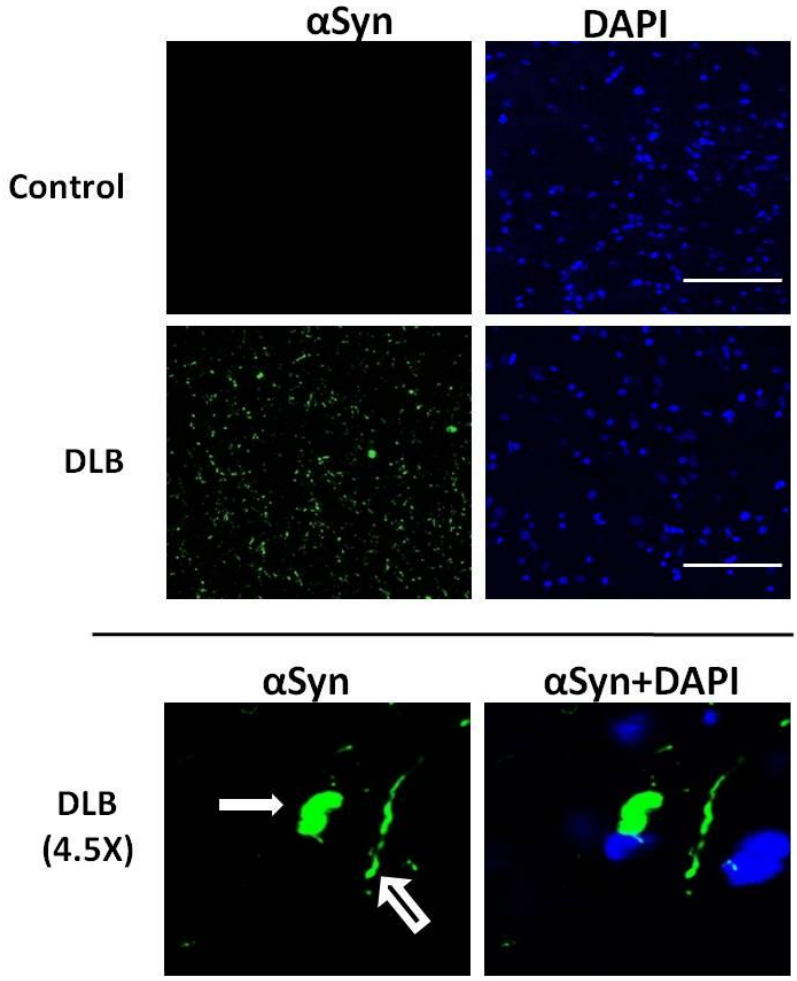
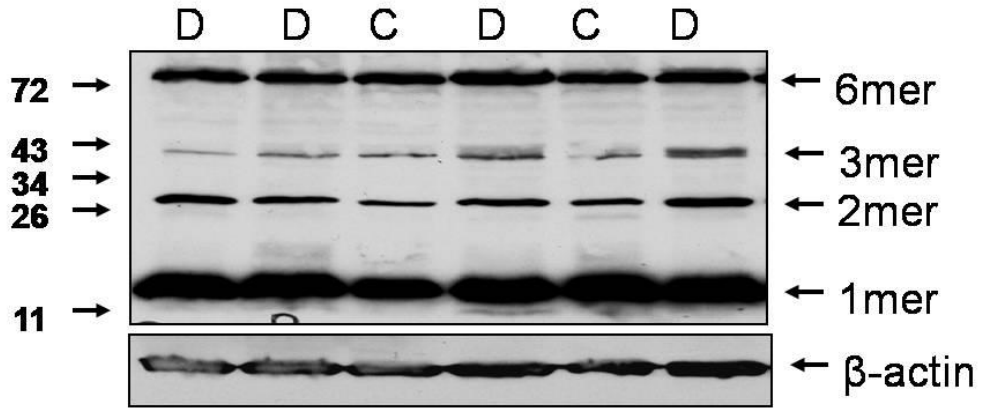
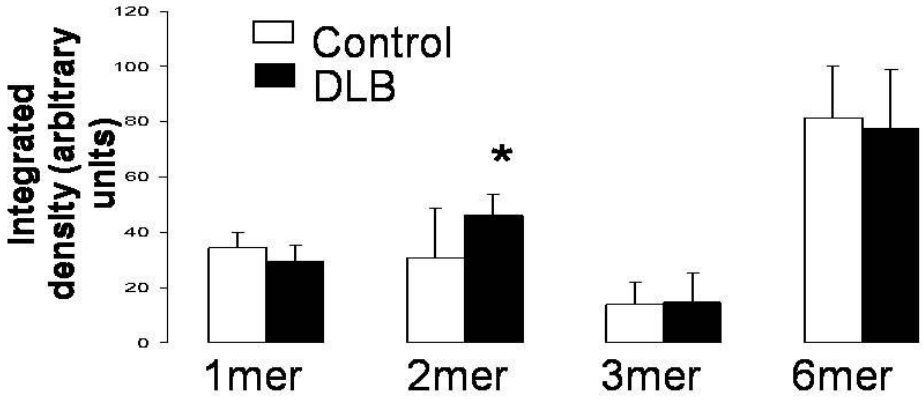


Figure 4.1 Immunohistochemistry reveals αSyn aggregates in the DLB frontal cortex that is not seen in age-matched controls. Representative confocal images

illustrating αSyn aggregates in the cortex of a DLB patient (D) in comparison to an age-matched control (C). Sections were mounted using a DAPI-containing mounting medium to visualize cell nuclei.





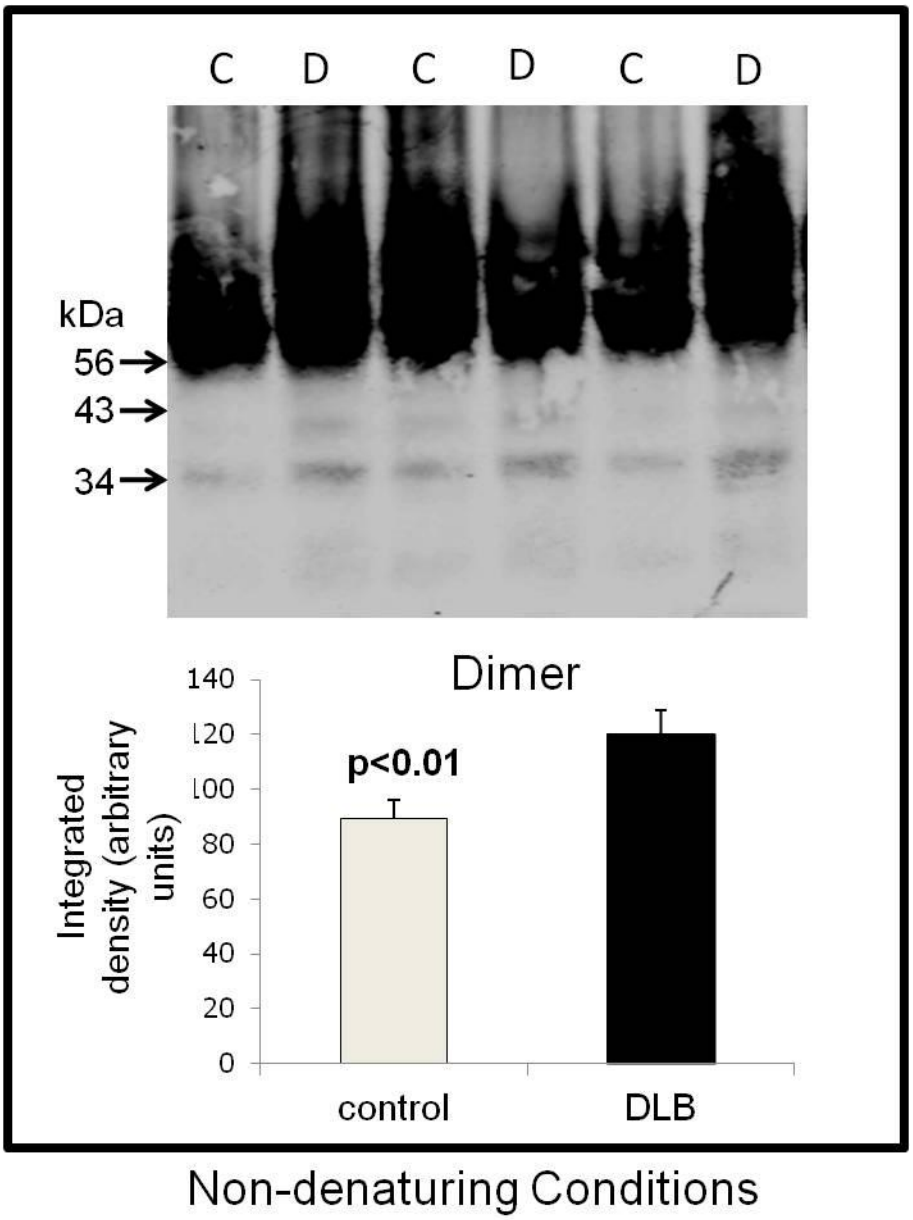


Figure 4.2 Increase in dimeric αSyn oligomers. Representative Western blot (top) detecting αSyn monomer (1mer) and oligomers (2-6mer) in soluble protein extracts from the frontal cortex of DLB patients (D) and age-matched controls (C). (Bottom) Densitometric analysis of immunoreactive bands comparing levels of individual αSyn species in frontal cortices from DLB patients (N=7) and age-matched controls (N=5). *: $p<0.05$ vs. control (ANOVA).

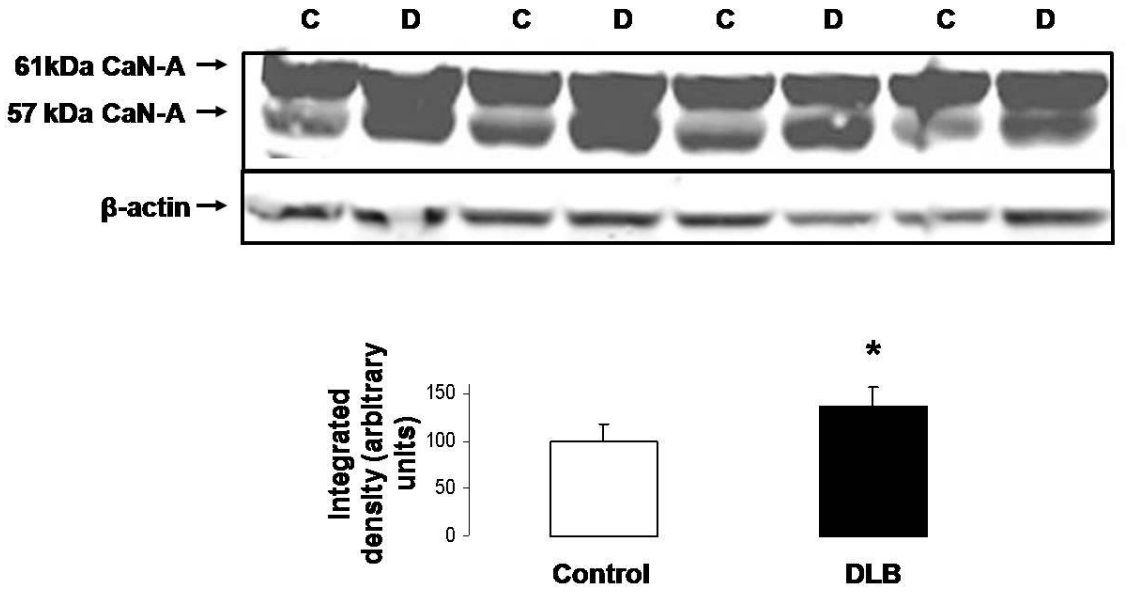


Figure 4.3 Truncation (activation) of CaN is increased in the DLB frontal cortex. (Top) Representative Western blot detecting CaN subunit A in protein extracts from frontal cortex of DLB (D) and age-matched controls (C). Membrane was stripped and re-probed for β-actin (loading control). (Bottom) Densitometric analysis of immunoreactive bands comparing CaN truncated subunit A levels in frontal cortices from DLB patients (N=7) and age-matched controls (N=5). *: $p<0.05$ vs. control (ANOVA).

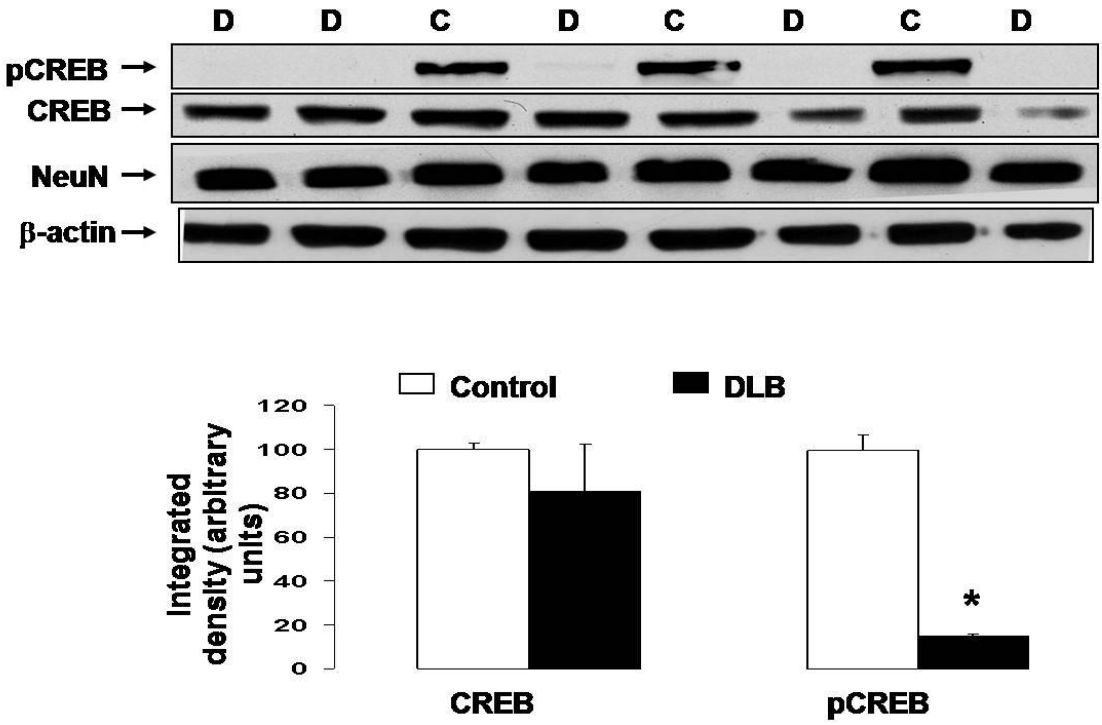
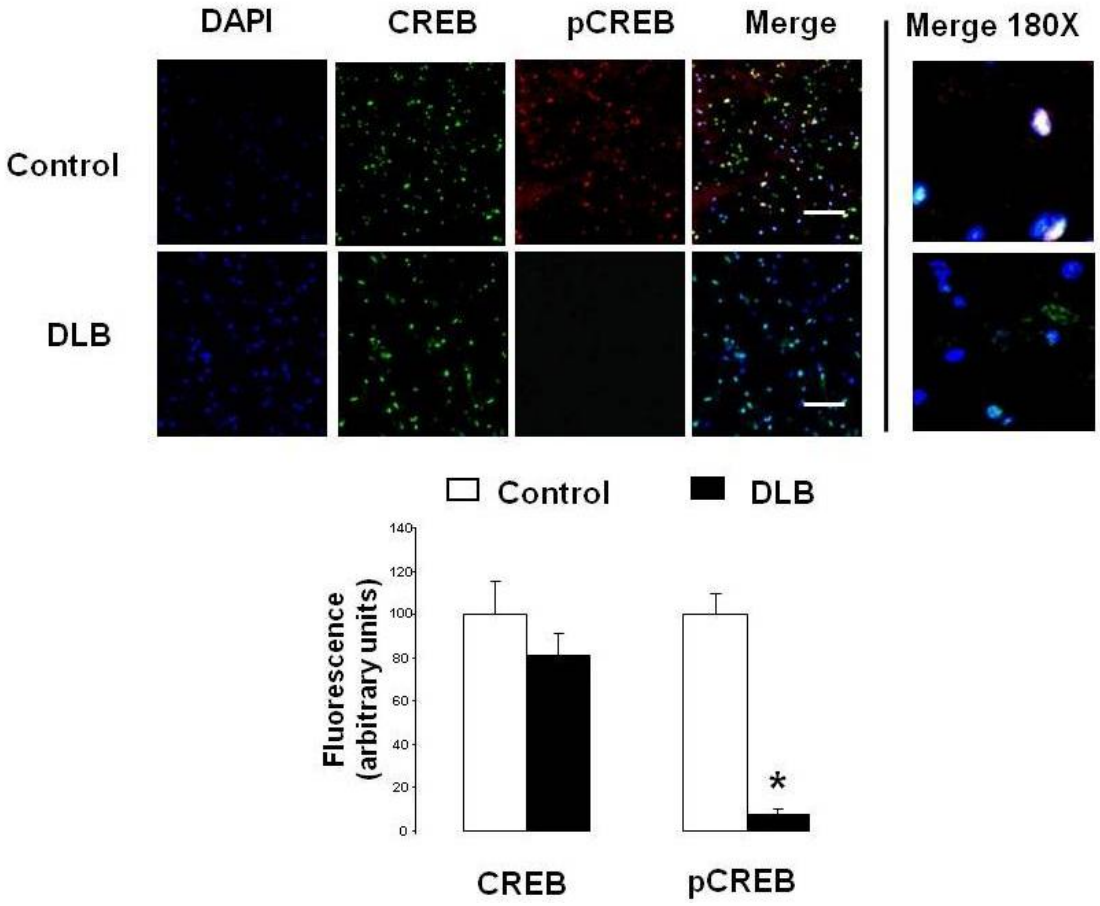


Figure 4.4 Phosphorylation (activation) of CREB is decreased in the DLB frontal cortex. (Top) Representative Western blot detecting pCREB in protein extracts from the frontal cortex of DLB (D) and age-matched controls (C). Membrane was stripped and re-probed for total CREB, NeuN (neuronal marker), and β-actin (loading control). (Bottom) Densitometric analysis of immunoreactive bands comparing CREB and pCREB levels in frontal cortices from DLB (N=7) and age-matched controls (N=5). *: $p<0.001$ vs. control (ANOVA).


DAPI
CREB
pCREB
Merge
Merge 180X
Control
DLB
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DLB
Fluorescence (arbitrary units)
140
120
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CREB
pCREB

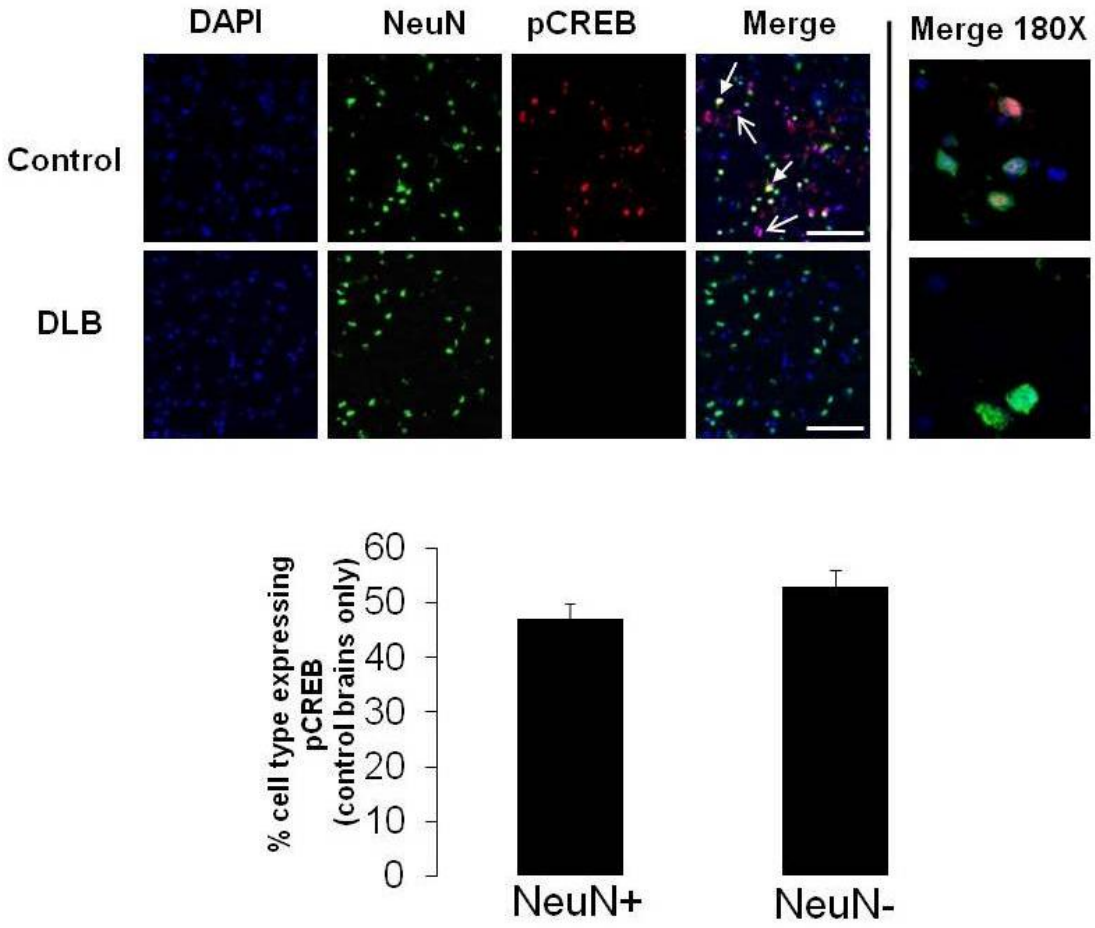

Figure 4.5 Immunohistochemistry confirms a reduction of pCREB in the DLB frontal cortex that affects both neuronal and non neuronal cells. Representative confocal images illustrating pCREB expression in the cortex of a DLB patient in comparison to an age-matched control. Sections were further co-stained for total CREB (upper two rows) or NeuN (a neuronal marker, bottom two rows) and mounted using a DAPI-containing mounting medium to visualize cell nuclei. Both neuronal ($NeuN^+$, solid-head arrows) and non neuronal ($NeuN^-$, open-head arrows) cells expressed detectable pCREB that was appreciably reduced in DLB for both cell types. The bar graph at the bottom illustrates the results from the image analysis of captured confocal images of

sections co-stained for pCREB and CREB. N=4 each DLB and control; *: p<0.01 vs. control (ANOVA).

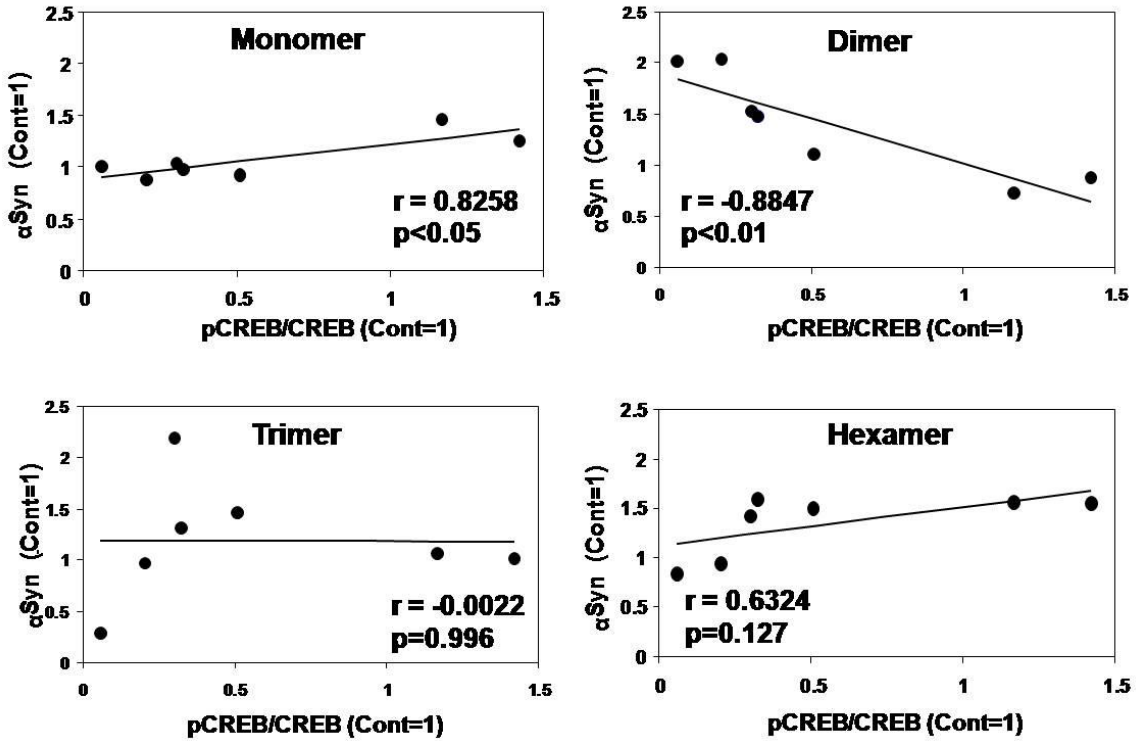

Figure 4.6 Levels of αSyn dimers are inversely correlated with levels of pCREB in the DLB frontal cortex. Correlation analysis comparing levels of monomer, dimer, trimer and hexamer species of αSyn to levels of pCREB (adjusted for CREB levels in the same sample) in the DLB frontal cortex. Levels for both αSyn species and pCREB/CREB ratio were expressed as fraction of the average of the corresponding value in age-matched controls, which was thus set equal to 1.

Discussion

These results provide evidence that there are mechanisms linking αSyn aggregates and signaling elements important for synaptic plasticity that may be at play in actual brains from humans affected by clinically diagnosed DLB. Specifically, in these

human DLB brains I found significantly higher levels of αSyn dimer (Fig. 4.2), higher levels of truncated, active CaN (Fig. 4.3), and lower levels of pCREB (Figs. 4.4 and 4.5). In addition, I found that levels of the αSyn dimer had a significant negative correlation with pCREB levels (Fig. 4.6). It is therefore prudent to hypothesize that αSyn oligomers, through affecting levels of elements such as CaN and pCREB that are intimately involved in the control of synaptic plasticity, may play a role in cognitive decline accompanying the progression of DLB, and possibly other synucleinopathies.

I could not directly measure CaN activity in the brain samples used in the present studies as its enzymatic activity, regardless of disease state, was extremely low and often indistinguishable from background values (not shown). Loss of phosphatase activity, and particularly CaN, is common in autopsy specimens where quick post-mortem oxidative deactivation of phosphatases cannot be practically prevented through timely cryopreservation (Wang, Culotta et al. 1996). However, based on past literature showing a selective and typical truncation of the subunit A upon CaN activation (Liu, Grundke-Iqbal et al. 2005), I was able to measure levels of truncated CaN as an indicator of CaN activation and found a significant increase in DLB tissue relative to control (Fig. 4.3). This finding is notable because it is known that activation of CaN is both Ca^{2+} and calmodulin dependent (Mansuy 2003), which corresponds with our earlier findings that synthetic oligomeric αSyn causes an increase in intracellular Ca^{2+} and CaN activation. Moreover, I observed a dramatic decrease in CREB phosphorylation in human cortex samples from patients affected by DLB as compared to age-matched controls (Figs 4.4 and 4.5). Such a decrease was correlated with increased levels of a specific αSyn dimeric oligomer (Fig. 4.6), suggesting that a similar mechanism linking oligomeric species of αSyn and decreased pCREB as previously observed in experimental models (Neugebauer et al., this issue) may be at play in the actual diseased human brain. Moreover, it is particularly notable that the dimeric species was

the only oligomeric species significantly higher in DLB brains relative to control (Fig. 4.2) and had the highest negative correlation with pCREB levels (Fig. 4.6) in that growing literature evidence pertaining to the Alzheimer's amyloid-beta peptide points to the dimers as being the most synaptic toxic oligomers of this particular amyloid protein (Jin, Shepardson et al. ; Shankar, Li et al. 2008; Mc Donald, Savva et al. 2010). With this in mind, even though I detected αSyn oligomers in brain specimens from age-matched control subjects (Fig. 4.2), no αSyn oligomeric species was statistically correlated to pCREB levels in the healthy human cortices (data not shown), suggesting that an additional factor, likely specific to the disease state, is necessary to render αSyn oligomers (particularly dimers) capable of affecting pCREB levels. What this putative factor may be is currently unknown; even though it is extremely tempting to speculate that a mechanism involving αSyn oligomers causing Ca^{2+} dyshomeostasis may be an attractive possibility that however awaits experimental confirmation.

Ca^{2+} dyshomeostasis leading to synaptic dysfunction has been shown to be one common event observed in neurodegenerative diseases (Mattson 2007); however the concurrent signaling events such as phosphatase or kinase level modulation has not been fully elucidated. Studies have shown opposing effects from amyloid aggregates on phosphorylation events (Price, Rockenstein et al. ; Yuan, Sun et al. ; Hashimoto and Masliah 2003; Reese, Zhang et al. 2008).This possibly could be explained by different experimental models or brain areas studied. However due to the predominant location of αSyn in the presynapse and its role in vesicle trafficking, it seems likely that pathological effects elicited by abnormally aggregated αSyn would lead to synaptic dysfunction. Our study points to one possible molecular mechanism through which the oligomeric species of αSyn affect these synaptic systems through its effects on CaN activation and decreased levels of pCREB. This is complemented by studies showing upregulation of CaN in aged brains and its correlation to the degree of cognitive impairments (Foster,

Sharrow et al. 2001; Foster 2007). Since DLB is a cognitive disorder, it is therefore tempting to speculate a direct correlation between αSyn oligomers and CaN hyperactivation in the pathology of DLB.

In conclusion, our findings illustrate molecular events that could mediate the impact of αSyn oligomers on synaptic dysfunction in the DLB human cortex, namely activation of CaN and associated dephosphorylation of CREB. Since these events precede overt cell death, it is conceivable that synaptic dysfunction can be reversible and thus amenable to pharmacological intervention. It is therefore prudent to suggest that CaN inhibition should be considered as a possible therapeutic strategy for DLB and other synucleinopathies.

Chapter 5: Seeding of alpha synuclein aggregation by nitrated alpha synuclein promotes the formation of stable oligomers with increased toxicity.

Abstract

Synucleinopathies such as Parkinson disease make up a group of neurodegenerative disorders characterized by Lewy Bodies which are insoluble protein aggregates formed through β-sheet interactions containing predominantly fibrils of αSyn protein. Soluble oligomeric intermediates of the fibrillation pathway have shown to be the most toxic species. One mechanism of toxicity of αSyn oligomers is through membrane destabilization which results in increased intracellular Ca^{2+} and altered cell signaling homeostasis as described in the previous chapters.

Oxidative and nitrative stressors have also been shown to be associated with synucleinopathies, but the exact mechanism of toxicity is not well understood. αSyn oligomers formed in response to nitration associate through covalent dityrosine

crosslinking, unlike the non β-sheet interaction that marks amyloid aggregates, thus suggesting the existence of substantial differences between oligomers formed by nitrated αSyn as compared to unmodified αSyn. The present study was therefore aimed at investigating structural and functional differences between oligomeric aggregates obtained from nitrated and non-nitrated αSyn. The results show that nitrated αSyn demonstrates conformational differences both on Western blot and AFM along with increased resistance to β-sheet disrupters. Unlike unmodified αSyn oligomers, nitrated αSyn oligomers have no impact on intracellular Ca^{2+} in SY5Y human neuroblastoma or on early expression of LTP and pCREB in rodent brain slices. However, decreased cell viability was observed in SY5Y cells after treatment with nitrated αSyn species for twenty-four hours. Notably, αSyn seeded with nitrated monomers (1:10) causes substantially greater toxicity than unseeded αSyn oligomers, which suggests that detrimental effects of nitrated αSyn in synucleinopathies may be exerted indirectly. Indeed, the present study suggests one causal mechanism for synuclein pathology being nitrative stress-related αSyn modifications initiating potent amyloid toxicity of otherwise normal αSyn.

Introduction

Synucleinopathies are a group of neurodegenerative disorders characterized by Lewy Bodies which are insoluble aggregates containing predominantly αSyn protein (Baba, Nakajo et al. 1998). All known synucleinopathies share a similar mechanism of neuropathology that involves a native monomeric αSyn protein structure rearranging to a β-sheet rich conformation followed by nucleation-dependent aggregation into soluble oligomers, protofibrils, fibrils, and finally Lewy body deposits (Jarrett and Lansbury 1993; Harper and Lansbury 1997; Makin and Serpell 2002). Within these different αSyn aggregated species, the oligomer and protofibril intermediates have shown to be the

most toxic (Sharon, Bar-Joseph et al. 2003; Olanow, Perl et al. 2004; Demuro, Mina et al. 2005).

Annular protofibrils can readily integrate into the cell membrane, perturbing membrane permeability, and creating ion dyshomeostasis such as increased intracellular Ca^{2+} levels (Ding, Lee et al. 2002; Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002; Kayed, Head et al. 2003; Zhu, Li et al. 2003; Demuro, Mina et al. 2005; Glabe and Kayed 2006). Evidence to show αSyn's ability to bypass cell membranes is shown by αSyn's presence in the CSF and plasma of PD patients (Borghi, Marchese et al. 2000; El-Agnaf, Salem et al. 2003), studies showing cultured neurons releasing αSyn in the medium (Lee, Patel et al. 2005), and fetal tissue grafted into the CNS of PD patients eventually developing αSyn aggregates (Kordower, Chu et al. 2008; Li, Englund et al. 2008). Other indications of protofibril toxicity is demonstrated in three missense mutations (A53T, A30P, and E46K) in the αSyn protein that are linked to rare forms of familial synucleinopathy PD and that form predominantly protofibril structures while having a significantly greater membrane destabilizing effects, suggesting this mechanism of toxicity by αSyn protofibrils as being a key event in PD pathology (Conway, Harper et al. 2000; Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002; Volles and Lansbury 2002).

Oligomer-induced ion dyshomeostasis such as rises in intracellular Ca^{2+} could profoundly affect neuronal function and eventually cause neuronal death through mechanisms such as hyperactivation of the phosphatase calcineurin (CaN, a critical regulator of Ca^{2+} signaling, synaptic plasticity, learning and memory, and cell survival) (Winder, Mansuy et al. 1998; White, Sullivan et al. 2000; Josselyn, Kida et al. 2004; Yang, Omori et al. 2004), causing CREB dephosphorylation (Winder, Mansuy et al. 1998; Nishi, Snyder et al. 1999; Josselyn, Kida et al. 2004). Dephosphorylation and

inactivation of this transcription factor causes a decrease in synaptic plasticity, long-term potentiation (LTP, the cellular basis of learning and memory), and learning and memory (Mansuy, Mayford et al. 1998; Schulz, Siemer et al. 1999; Malleret, Haditsch et al. 2001).

However, some αSyn oligomeric species, such as those formed in a nitrative or oxidative environment, are derived from an off-pathway aggregation and may thus differ both structurally and functionally from oligomers formed by unmodified αSyn. Nitrative conditions cause covalent dityrosine crosslinking that bypasses the nucleation-dependent lag phase, thus leading to a much quicker formation of oligomers (Zhou, Long et al. ; Yamin, Uversky et al. 2003) that have increased stability (Souza, Giasson et al. 2000; Norris, Giasson et al. 2003; Uversky, Yamin et al. 2005), and reduced propensity to form fibrils (Souza, Giasson et al. 2000; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). Importantly, dityrosine crosslinking can also occur between preformed aggregates, but because these aggregates are already in parallel, register conformation, their structure differs from that of aggregates formed through dityrosine crosslinking between monomers in that these latter do not involve β-sheet-driven interactions and consequently in-register conformation (Souza, Giasson et al. 2000; Norris, Giasson et al. 2003; Ruf, Lutz et al. 2008).

Evidence has revealed an association between nitrative/oxidative stress and synucleinopathies. For instance, nitrative stress and synucleinopathy incidences increase with age and both elicit insult on the post-mitotic neurons (Choi, Zhang et al.). Furthermore, it is thought that dopaminergic neurons are most susceptible during PD because of the oxidative environment created through dopamine metabolism (Dauer and Przedborski 2003). More directly, oxidative and nitration stress have also been observed

on Lewy body deposits in postmortem PD and DLB brains (Duda, Giasson et al. 2000; Giasson, Duda et al. 2000; Gomez-Tortosa, Gonzalo et al. 2002; Dalfo, Portero-Otin et al. 2005). Other studies have shown nitrative stress being the mediator between the synergism of environmental stimuli (such as inflammatory-stimulating agents or herbicides) and Tg αSyn animal models (Peng, Oo et al. ; Yu, Xu et al. ; Gao, Kotzbauer et al. 2008). Another recent study has shown using an internalization signaling peptide on αSyn that nitrated αSyn is more toxic than non-nitrated αSyn (Yu, Xu et al.); however, the same authors point out that the toxicity was diminished when the internalization tag was removed, implying that the mechanism of this toxicity doesn't involve membrane instability. Indeed, even though αSyn's most commonly-known mechanism of toxicity is through membrane destabilization (Lashuel, Hartley et al. 2002; Volles and Lansbury 2003), not all αSyn oligomers share this characteristic (Uversky ; Fink 2006).

Notably, nitrated and non-nitrated αSyn can still influence each other's aggregation pathway as studies have shown that even substoichiometric amounts of the heterogeneous oligomers created through oxidative and nitrative modifications lead to fibril inhibition of non-modified αSyn (Zhou, Long et al. ; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). Such reported substoichiometric effects of nitrated αSyn on non-nitrated αSyn are of particular significance to the neuropathology of synucleinopathies because it has been shown that only a fraction of the total αSyn pool is nitrated during nitrative stress (Hodara, Norris et al. 2004). This suggests that even a minor fraction of the αSyn pool that is nitrated under condition of oxidative stress may have a dramatic impact on the unmodified αSyn structure and toxicity; consequently, there may exist an indirect association between protein nitrative events and neurodegeneration that implicates oxidative stress being a causative factor into synuclein-pathology. On these bases, the present study investigated toxicity of nitrated

αSyn aggregated species per se as well as the toxicity of unmodified αSyn oligomers formed through seeding with substochiometric amounts of nitrated αSyn aggregates.

Results

Recombinant αSyn was nitrated using TNM either before or after oligomerization in order to compare nitration effects on monomer, nitration effects on pre-formed oligomer, and non-nitrated αSyn. Structural differences between the nitrated and non-nitrated species were assayed using Western blot and AFM analyses (shown in Figure 5.1). Nitrated monomer (Nmono) and αSyn oligomer were first tested on Western probed with anti-αSyn. Nmono had multiple bands even though it was not subject to the oligomerization procedure (see Methods), implying that nitration per se induces oligomerization of αSyn monomer. Also, even though both samples had multiple bands (reflecting the presence of oligomeris species), the apparent molecular weight of the bands in the Nmono sample was shifted as compared to αSyn oligomer's bands, suggesting that the two structures were different resulting in different electrophoretic migration patterns. Specifically, Nmono species migrate faster down the electrophoretic gel than unmodified αSyn oligomer, implying Nmono has a tighter, more rigid structure (Western blot shown in Figure 5.1A).

Nmono and αSyn oligomer Western blots were also probed with anti-NitroSyn. As expected, αSyn oligomer did not reveal any bands; however, Nmono had bands at the same location as the bands revealed by probing the gel with anti-αSyn, confirming proper nitration of the αSyn recombinant protein. Nmono and αSyn oligomer blots were further probed with anti-NitroTyr; as expected, αSyn oligomer did not show any immunoreactivity. On the other hand, Nmono revealed bands at the same location as observed in the previous two blots, suggesting that nitration occurred at the Tyr residues

of αSyn (Blot shown in Figure 5.1A). Western blot analysis was also done on nitrated pre-formed αSyn oligomers (Noligo) and probed with anti-NitroSynuclein to evaluate whether there were nitration differences between Nmono and Noligo; the blot revealed bands of similar apparent molecular weight, even though Nmono revealed increased higher molecular weight species than Noligo. Also, Nmono had more intense staining than Noligo, suggesting more nitration had occurred on Nmono than Noligo (Blot shown in Figure 5.1B). AFM analyses on Nmono, Noligo, and αSyn oligomer revealed structural differences as well. For instance, the αSyn oligomer image showed primarily small spherical structures, the Nmono image revealed bigger globular structures, while the Noligo image returned a mixture of both (AFM shown in Figure 5.1C).

To further investigate structural differences among the three αSyn species, chaotropic treatment with known β-sheet disrupters acetonitrile and hexa-fluoro-isopropranol (HFIP) was performed and compared to non-treated samples by Western blot probed with anti-αSyn. Nmono and Noligo did not demonstrate band differences before and after chaotropic treatment, whereas αSyn oligomers had increased monomers and decreased aggregates after chaotropic treatment. This result suggests that molecular interactions other than β-sheet (e.g., dityrosine crosslinking between individual αSyn proteins) occur in the aggregates of the nitrated species (Blot and densitometry analysis shown in Figure 5.2).

To investigate possible differences in cellular effects amongst the different αSyn species, intracellular Ca^{2+} levels were measured on fura-2-loaded SY5Y cells after treatment with 4 μM of the different αSyn species. Consistent with our previous results (Chapter 3), αSyn oligomers caused an increase in intracellular Ca^{2+} immediately after treatment (Figure 5.3A). On the other hand, treatment of Nmono did not increase intracellular Ca^{2+} (Figure 5.3B) and Noligo evoked an increase in intracellular Ca^{2+} levels

immediately after treatment, but not as robust as αSyn oligomer (Figure 5.3C). While this latter observation may indicate the presence of residual non-nitrated oligomers in the Noligo sample, collectively these experiments suggest that it is unlikely that nitrated species induce cell toxicity through increased intracellular Ca^{2+}.

Neuronal cell effects of nitrated αSyn was further investigated on LTP expression that was measured on rat brain slices that were treated with 0.5 μM of the different αSyn species. Consistent with our previous results (Chapter 3), αSyn oligomers completely abolished LTP expression. Nmono and Noligo did not abolish LTP expression at first, but its duration was shorter than that recorded in non-treated, control slices (Figure 5.4A). On these same brain slices, pCREB/CREB was measured at the end of the recording period using Western blot analysis. Also confirming our previous results, pCREB levels were decreased in the αSyn oligomer treated slices; however, pCREB levels were unchanged after Nmono or Noligo treatment, implying no effect on pCREB levels during the duration of the sixty minute recording (Figure 5.4B).

Next, I measured possible structural differences between αSyn aggregated in the presence of substochimetric amounts (seed) with Nmono (Nseeded, 1:10) in comparison with αSyn aggregated under non-seeded conditions. Both samples were stirred five days (a time period usually resulting in abundant fibril formation) and evaluated using Western blot probed with anti-αSyn and AFM analyses. Western blot was performed using two different gel concentrations, 10% and 6% acrylamide, so as to best separate low and high molecular weight aggregates, respectively. Non-seeded αSyn had higher molecular weight bands displayed in the 6% gel and decreased lower molecular weight bands in the 10% gel, implying expected fibril formation in this sample. On the other hand, the Nseeded sample had increased oligomeric species as detected in the 10% gel and much less higher molecular weight species revealed on the 6% gel as compared to non

seeded αSyn, implying the seeding with Nmono caused inhibition of αSyn fibrillation (Figure 5.5A). AFM analysis of these two samples confirmed the Western blot results in that the non-seeded sample primarily contained fibrillar αSyn while the Nseeded sample was composed of smaller spherical species (Figure 5.6B). This is consistent with literature demonstrating that seeding αSyn with nitrated αSyn results in the formation of stable oligomers that do not further aggregate into fibrils.

Cell viability was measured on SY5Y cells twenty-four hours after treatment with 4 μM of the αSyn species. As compared to untreated control cells, αSyn oligomer-treated cells had ~55% viability, Nmono-treated cells had ~70% viability, Noligo-treated had ~50% viability, Nseeded-treated had ~15% viability, and αSyn fibrils had ~95% viability (figure 5.6A). These results suggest that all αSyn aggregated species induced cell toxicity, with the exception of αSyn fibrils. Notably, treatment with Nseeded produced the highest degree of toxicity in the SY5Y cells. To determine the concentration of Nseeded that elicits the same level of toxicity as 4 μM αSyn oligomers, I treated cells with increasing doses of Nseeded (ranging 0.5 to 4 μM) and observed that the toxicity induced by Nseeded applied at 1-2 μM was comparable to that induced by 4 μM αSyn oligomers (indicated by a red line in Figure 5.6A,B).

Intracellular Ca^{2+} levels were measured on fura-2 loaded SY5Y cells after treatment with different concentrations of Nseeded αSyn. Measuring levels of intracellular Ca^{2+} after adding 4 μM Nseeded was not achievable because the fura-2 dye was immediately released outside of the cells upon treatment (Figure 5.7A). Similar results were obtained after treatment with 2 μM Nseeded (Figure 5.7B). One possibility to explain these results is that concentrations of 2 and 4 μM Nseeded cause immediate membrane destabilization to the cells, thus leading to loss of intracellular Fura-2 dye and eventually death of cells. On the other hand, treatment of SY5Y cells with 1 μM Nseeded

did not elicit an increase of intracellular Ca^{2+} (Figure 5.7C), implying a concentration higher than 1 μM but lower than 2 μM of Nseeded αSyn being at least equivalent to the effect elicited by 4 μM αSyn oligomers. This is consistent with the results observed in the cell viability experiments, where treatment with Nseeded resulted 2 to 4 times more toxic αSyn oligomers. LTP studies were also attempted using Nseeded treatment on rat brain slices, but no synaptic output could be measured, suggesting that treatment with Nseeded was more toxic than treatment of αSyn oligomers (data not shown). Collectively, these experiments suggest Nseeded αSyn oligomers are substantially more toxic than non-seeded αSyn oligomers.

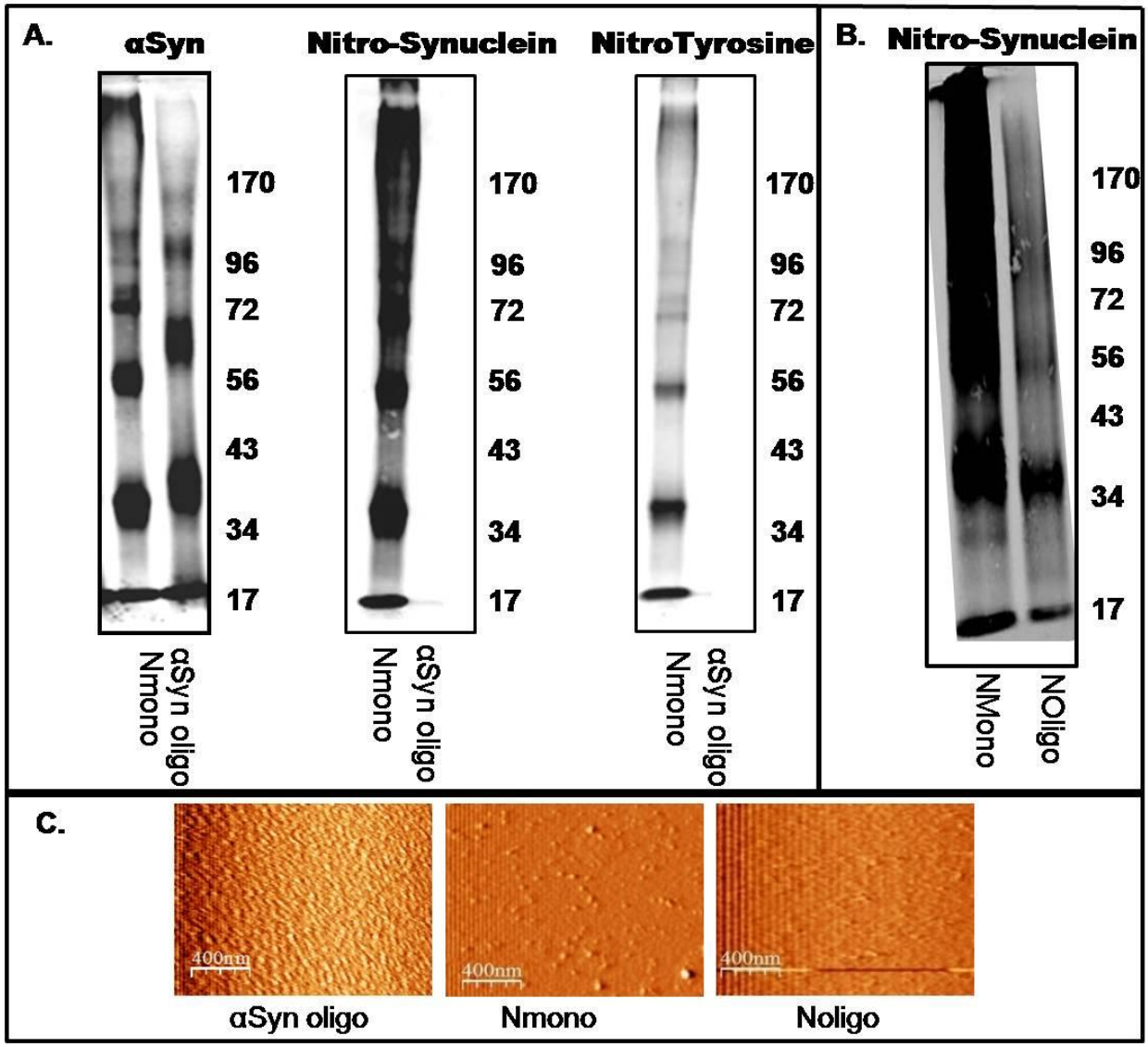

Figure 5.1: αSyn nitrated by TNM confirmed using Western blot and AFM. Representative Western blots (A) detecting αSyn oligomers and/or Nmono using antibodies for αSyn, NitroSyn, and NitroTyr. Representative Western blots (B) detecting Nmono and Noligo using antibody for NitroSyn. AFM images (C) of αSyn oligomers, Nmono, and Noligo.

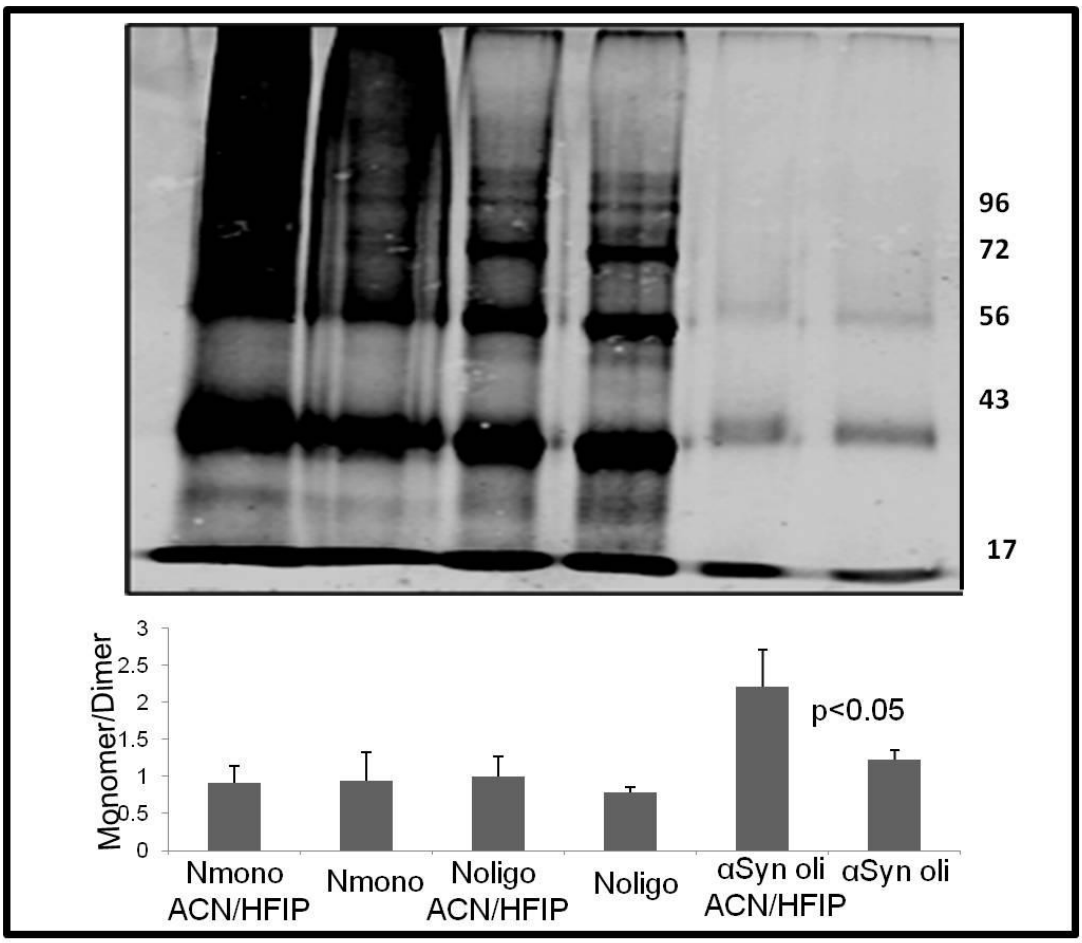

Figure 5.2: Nitrated αSyn resistant to β-sheet disrupting compounds acetonitrile and HFIP. (Top) Representative Western blot detecting αSyn oligomers, Nmono, and Noligo with and without treatment of acetonitrile and HFIP. (Bottom) Densitometric analysis of immunoreactive bands comparing treated and untreated samples. *: $p<0.05$ treated vs. untreated of αSyn oligomers (ANOVA).

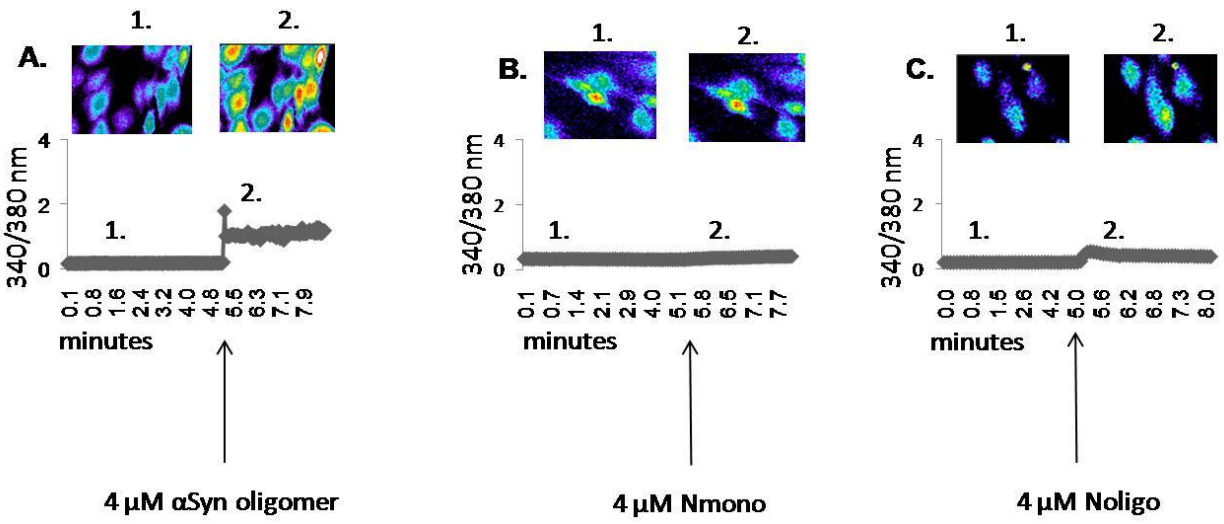

Figure 5.3: Nitrated αSyn has no effect on intracellular Ca^{2+} levels as compared to αSyn oligomers on SY5Y cells. Representative images illustrating intracellular Ca^{2+} stained with fura-2 and graphs depicting fluorescent readout of 340/380 nm after treatment with αSyn oligomers (A), Nmono (B), and Noligo (C). N=30.

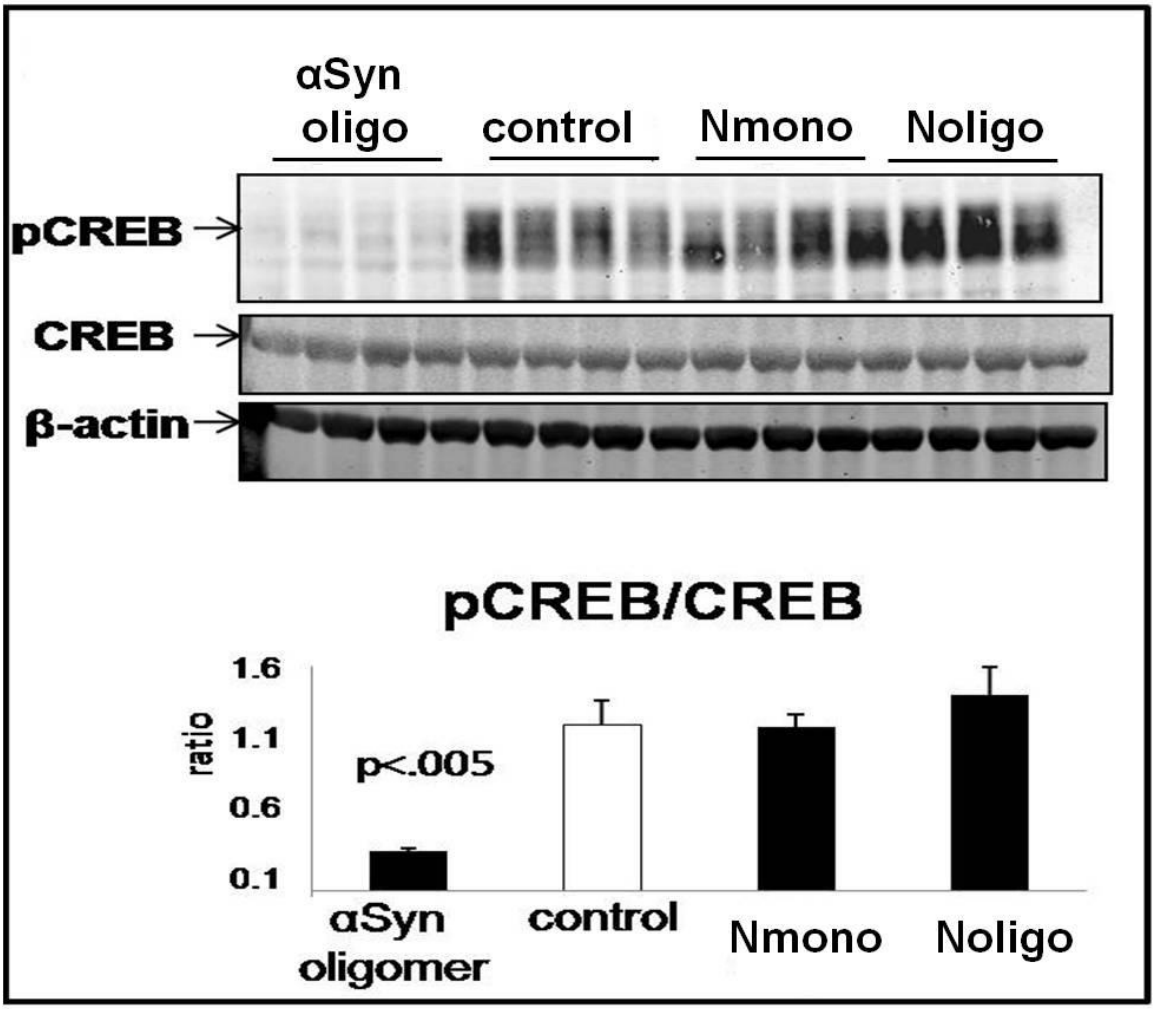

Figure 5.4: Nitrated αSyn shortens duration of LTP expression but has no effect on pCREB levels after sixty minutes. Graph (A) representing percent amplitude of EPSCs after treatment with αSyn oligomers, Nmono, or Noligo. Western blot (B) measuring pCREB/CREB levels on soluble protein fraction of rat brain slices used for LTP experiment.

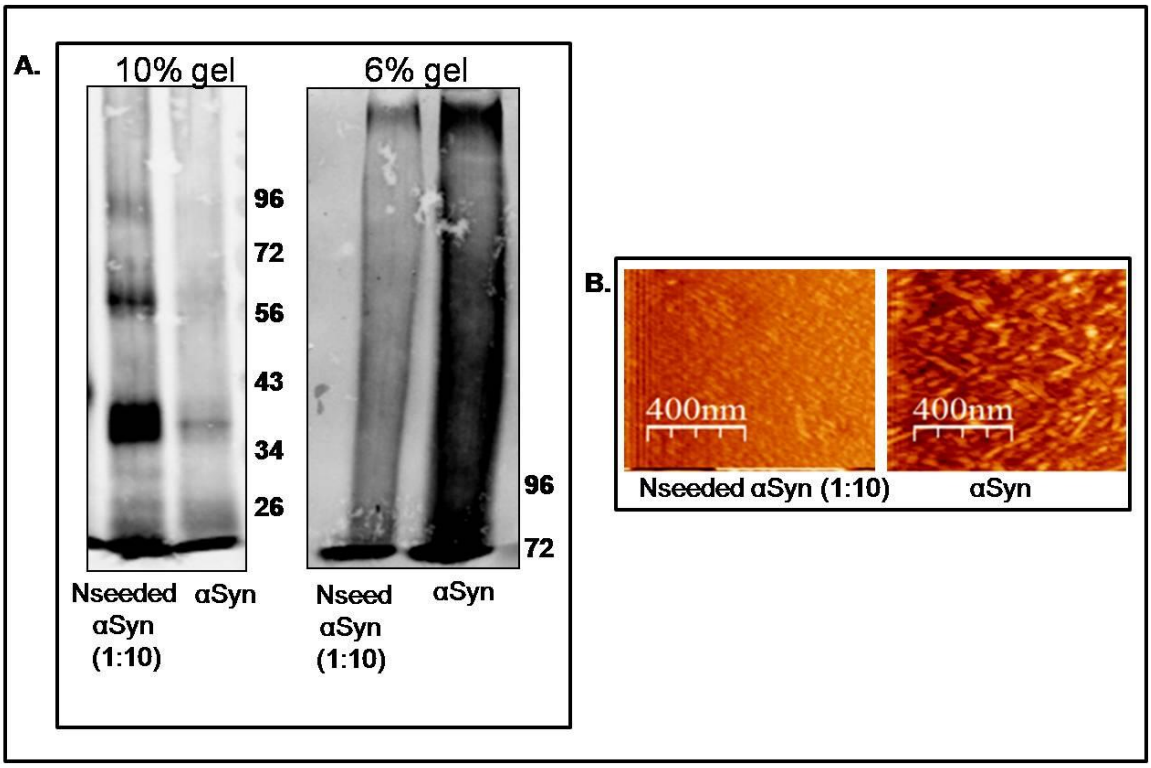

Figure 5.5: Seeding αSyn with NαSyn (1:10 NαSyn:αSyn) and stirred 5 days leads to different structure from αSyn stirred alone 5 days. Western blots (A) of Nseeded and αSyn after five days stirring on 6% and 10% acrylamide gels. AFM images (B) of Nseeded and αSyn after five days stirring.

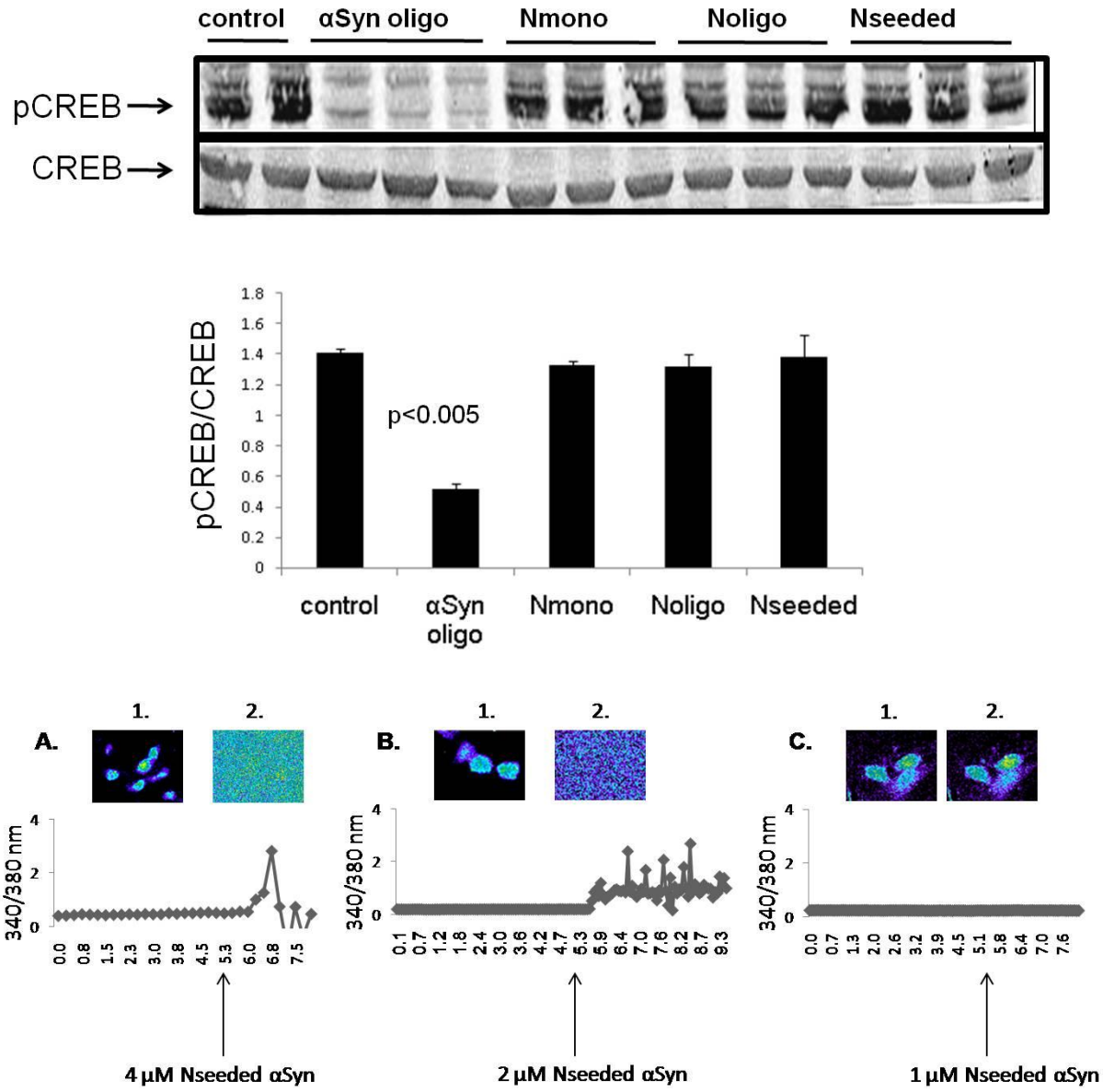

Figure 5.7: 4 and 2 µM Nseeded αSyn causes release of fura-2 from cells while 1 µM Nseeded shows no increase in intracellular Ca^{2+} on SY5Y cells. Representative images illustrating intracellular Ca^{2+} stained with fura-2 and graphs depicting fluorescent readout of 340/380 nm after treatment with 4 µM Nseeded two sequential times (A), 2 µM Nseeded (B), and 1 µM Nseeded (C). N=30.

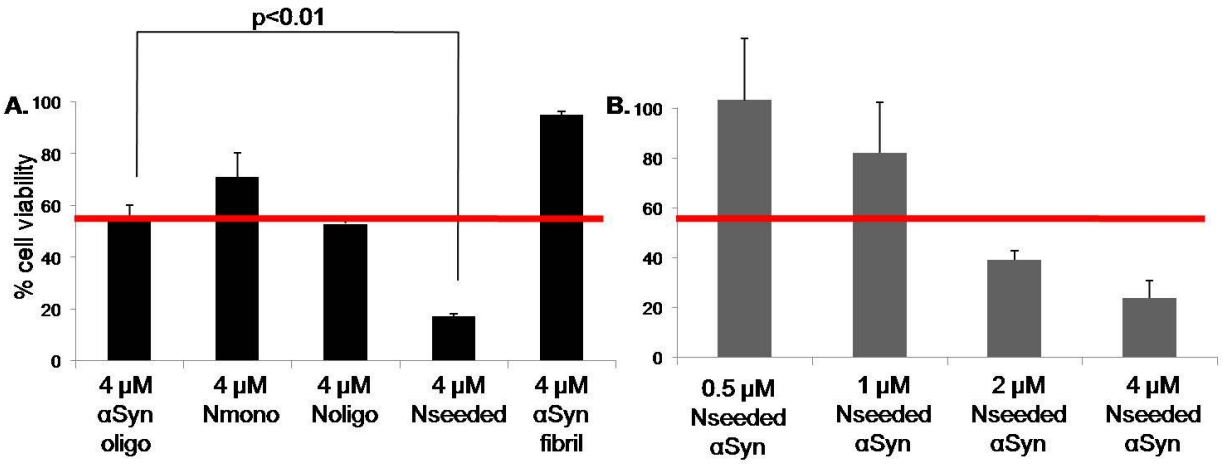

Figure 5.6: Nitrated αSyn show decreased cell viability after twenty-four hours, but Nseeded most toxic. Graphical representation (A) of cell viability using MTS after twenty-four hours treated with αSyn oligomer, Nmono, Noligo, Nseeded, or fibrils. Graphical representation (B) of cell viability using MTS of 0.5, 1, 2, or 4 µM Nseeded αSyn. N=3.

Discussion

This study provides evidence that there are structural and functional differences between nitrated αSyn and non-nitrated αSyn. Furthermore, this study demonstrates profound differences of Nseeded αSyn and non-seeded αSyn after five days stirring both structurally and functionally. Structurally, it is known that covalent modifications such as dityrosine crosslinking results in conformations different from amyloid oligomers formed through β-sheet interactions (Ruf, Lutz et al. 2008). This is also demonstrated in Figure 5.1A on the anti-αSyn Western blot illustrating bands from the Nmono sample migrate faster down the electrophoretic gel than αSyn oligomer, implying conformational changes of Nmono to a more rigid and stable structure. Also, Figure 5.1C demonstrates

structural differences amongst the αSyn species in that the αSyn oligomer image showed primarily small spherical structures, the Nmono image revealed bigger globular structures, while the Noligo image returned a mixture of both, demonstrating conformational differences of the oligomeric species that is presumably caused by covalent crosslinking in the nitrated species relative to β-sheet interactions in αSyn oligomer. It is interesting that the Western blot shown in Figure 5.1B demonstrates both greater anti-NitroSynuclein staining and higher molecular weight bands in the Nmono as compared to Noligo sample. It is conceivable that the monomer conformation of αSyn could possibly have increased Tyr availability for nitration, causing nitration-influenced acceleration of oligomerization, while the pre-formed oligomers are in a parallel, in-register stacked conformation with Tyr grouped together causing limited nitration (Ruf, Lutz et al. 2008). However, it is compelling that the Nmono and Noligo bands are at the same location on the Western blot if their conformations differ. Perhaps the covalent effects are more profound than β-sheet interactions, causing increased stability and rigidity. Indeed, it is documented that off-pathway oligomeric αSyn species have increased stability resulting from covalent interactions (Souza, Giasson et al. 2000; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). This is also demonstrated in Figure 5.2 where the nitrated species show increased resistance to β-sheet disrupters.

It is also known that the structural changes caused by dityrosine crosslinking can result in deleterious effects on αSyn's normal function in vesicle trafficking (Hodara, Norris et al. 2004), but not much is known about neurotoxicity as compared to unmodified αSyn oligomers. The present study shows functional differences with αSyn oligomer treatment on intracellular Ca^{2+} levels, LTP expression, and CREB phosphorylation. For example, Figure 5.3B demonstrates no increase of intracellular Ca^{2+} after treatment with Nmono, and Figure 5.3C shows only a limited increase of

intracellular Ca^{2+} after treatment with Noligo, which is in contrast with αSyn oligomer treatment that results in a significant increase of intracellular Ca^{2+} as shown in Figure 5.3A. One explanation for differences in intracellular Ca^{2+} elicited by the nitrated αSyn species is that these species have conformational differences from αSyn oligomers through covalent bonding interactions, as demonstrated in the Western blot and AFM analyses shown in Figures 5.1 and 5.2. Such covalent bonds between individual αSyn proteins would produce aggregates that do not possess structural and functional amyloid features since their interaction is not driven by the formation and anti-parallel juxtaposition of β-sheets that is the core feature of amyloid aggregates.

Nmono and Noligo did not abolish LTP expression at first, but the duration of expression was shorter than that recorded in non-treated, control slices (Figure 5.4A). One possible explanation for decreased duration of LTP expression is that the nitration efficiency of the samples could possibly not be complete, and amyloid aggregation of the non-nitrated αSyn portion in the sample could be seeded by the nitrated portion to form toxic oligomers at a lower concentration. On the other hand, the nitrated species did not affect pCREB levels sixty minutes after treatment as shown by Western blot (Figure 5.4B). Going with the idea of inter-seeding of the nitrated samples, it is probable that sixty minutes was not a sufficient time to elicit effects on CREB dephosphorylation since this event is downstream of oligomer-induced decreased LTP expression. Nonetheless, further studies should be done on brain slices using longer time periods to determine if effects on pCREB could eventually be observed. In addition, isolation of nitrated αSyn from potentially non-nitrated αSyn using affinity chromatography could be performed to ensure purity of the samples. The same phenomenon of intra-sample seeding by nitrated αSyn of toxic oligomers from residual non-nitrated αSyn could explain the cell viability data observed after twenty-four hour treatment (Figure 5.6A). The toxicity

observed in the nitrated αSyn samples at these longer time points complements the idea that incomplete nitration (leading to nontoxic nitrated αSyn aggregates but sparing residual αSyn molecules) would in time promote the formation of exceptionally toxic non-nitrated αSyn oligomers (however seeded by nitrated αSyn) that would induce toxicity even at nominal concentrations much lower than non-seeded αSyn oligomers.

To address this important point, the effects of Nseeded αSyn were evaluated using Nmono to seed αSyn at a (1:10) stoichiometric ratio. Figure 5.5 shows, using both Western blot and AFM analyses, that seeding αSyn with Nmono results in oligomeric species that did not further aggregate to form fibrils. These results confirm prior studies revealing that fibrillation of nitrated αSyn-seeded αSyn is inhibited and stable oligomers are primarily formed (Yamin, Uversky et al. 2003). However, little is known whether these stabilized oligomers are more or less toxic than αSyn aggregates formed in the absence of seeding with nitrated species. Figure 5.6A shows that Nseeded αSyn is more toxic than αSyn oligomers after twenty-four hours treatment in SY5Y cells. Indeed, investigations testing concentrations ranging from 0.5 to 4 μM demonstrate that the toxicity elicited by Nseeded at 1-2 μM is similar to that induced by 4 μM non-seeded αSyn oligomers (Figure 5.6B). Similar results were observed in the Ca^{2+} imaging experiments (Figure 5.7), where 2 and 4 μM Nseeded αSyn oligomers induced an immediate release of the intracellular fura-2 dye from SY5Y cells, thus suggesting that prompt cell membrane damage had occurred. Indeed, the cells visualized using DIC microscopy ruptured and were no longer visible at the same time the Fura-2 fluorescence diffused in the media, further suggesting that membrane destabilization and cell death had been promptly elicited after the addition of 2-4 μM Nseeded αSyn oligomers (data not shown).

This evidence of increased toxicity of αSyn oligomers formed through seeding with nitrated αSyn is highly significant to the yet unclear mechanisms underlying the etiology of αSyn neuropathology and the reported impact thereupon of oxidative/nitrative cell stress. Collectively these data suggest that an oxidative stress-promoted nitration even of a small fraction of the αSyn pool may seed the formation of exceptionally toxic oligomers from the unmodified αSyn pool. The resulting tritrated αSyn-seeded αSyn oligomers would be more stable than non-seeded αSyn aggregates and would not as quickly be detoxified by further aggregation into fibrils. This scenario would result in profound neurotoxicity, especially in those neurons whose metabolism is prone to generate high ambient levels of free radicals such as the dopaminergic neurons in the substantia nigra, one of the CNS regions most vulnerable in PD.

Chapter 6: Conclusion

This project focuses on underlying molecular events in synucleinopathies such as PD and DLB that involve toxic oligomeric assemblies of αSyn. Specifically, this project provides evidence for a novel pathway centered on CaN hyperactivation and concomitant CREB dephosphorylation, linking αSyn oligomers with synaptic dysfunction. Furthermore, this project demonstrates an association between synucleinopathies and oxidative/nitrative stress. Even though these two molecular events of oxidative/nitrative stress and synaptic dysfunction in synucleinopathies are separate, they have a compelling association that could be key to synucleineogenesis.

Like other disordered proteins, αSyn changes conformation relative to its environment. For instance, during pathological amyloidogenesis αSyn transitions conformation into a β-pleat structure followed by nucleation-dependent aggregation whereas the oligomeric species are the most toxic. One key mechanism of toxicity is

membrane destabilization resulting in ion dyshomeostasis such as increased Ca^{2+} (Lashuel, Hartley et al. 2002; Kayed, Head et al. 2003; Zhu, Li et al. 2003; Demuro, Mina et al. 2005; Glabe and Kayed 2006), but the downstream effects are not fully understood. Work previously done in our and our collaborators' laboratories have shown that oligomeric αSyn causes an increase in intracellular Ca^{2+}, CaN activation, decrease in pCREB, abolished LTP expression, and impaired memory in a CaN-dependent fashion *in vitro, ex vivo*, and *in vivo*. Notably, these intracellular signaling events were elicited from extracellularly applied αSyn. From the observation that αSyn oligomers cause an increase in intracellular Ca^{2+}, it is conceivable that these oligomers gain access to the cytosol through the membrane in order to elicit intracellular toxicity. This is complemented by studies revealing amyloid oligomer's ability to destabilize membranes through a pore-forming mechanism (Ding, Lee et al. 2002; Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002; Kayed, Head et al. 2003; Zhu, Li et al. 2003; Demuro, Mina et al. 2005; Glabe and Kayed 2006). Evidence in human disease to show αSyn's ability to bypass cell membranes is shown by αSyn's presence in the CSF and plasma of PD patients (Borghi, Marchese et al. 2000; El-Agnaf, Salem et al. 2003) and fetal tissue grafted into the CNS of PD patients eventually developing αSyn aggregates (Kordower, Chu et al. 2008; Li, Englund et al. 2008).

To extend these findings of αSyn oligomer-elicited effects on intracellular signaling involving CaN activation and decreased pCREB to the actual diseased human brain, my project began by testing postmortem DLB frontal cortices compared to age-matched controls in which I discovered significantly higher levels of αSyn dimer, higher levels of truncated/active CaN, and lower levels of pCREB. In addition, I found that levels of the αSyn dimer had a significant negative correlation with pCREB levels. Similar to the *in vitro*, *ex vivo*, and *in vivo* evidence in Chapter 3, this evidence

demonstrated αSyn oligomers, through affecting proteins that are intimately involved in the control of synaptic plasticity, CaN and pCREB, may play a role in cognitive decline accompanying the progression of DLB, and possibly other synucleinopathies.

CaN activation was determined by measuring levels of the truncated subunit A, which is an event during CaN activation based on past literature (Liu, Grundke-Iqbal et al. 2005). I could not directly measure CaN activity in the brain samples because its enzymatic activity, regardless of disease state, was extremely low. It is known that loss of phosphatase activity is common in autopsy specimens where post-mortem oxidative deactivation of phosphatases cannot be prevented during cryopreservation (Wang, Culotta et al. 1996). Even though this approach is indirect, it complements the prior studies from Chapter 3 showing αSyn oligomer-induced and Ca^{2+}-mediated CaN activation *in vitro, ex vivo*, and *in vivo*. Moreover, CaN activation provides a connection between αSyn oligomers and decreased pCREB levels seen in postmortem DLB brains using Western blot and IHC. However, other factors could be at play as well, such as decreases in kinases but by evaluating the *in vitro, ex vivo*, and *in vivo* evidence in Chapter 3 with the human postmortem results in Chapter 4, it is prudent to conclude that CaN has an impact on pCREB levels in DLB brains. It is also notable that the dimer was the only oligomeric species that was significantly higher in DLB brains and had a significantly negative correlation with pCREB because recent literature evidence pertaining to the Alzheimer's amyloid-beta peptide points to the dimers as being the most toxic oligomers of this particular amyloid protein (Jin, Shepardson et al. ; Shankar, Li et al. 2008; Mc Donald, Savva et al. 2010). By using a variety of tools including cells, rodent brain slices, animal models, and postmortem tissue, it is more reliable to pinpoint exact mechanisms of disease pathology by first directing an answer to one question to

be tested through *in vitro, ex vivo*, and *in vivo* systems and then validating the scientific findings from the prior systems with examples in human disease.

However, biomedical research can also work in the opposite direction by making an observation in human disease and then testing mechanistic reasons on the bench. For example, synucleinopathies are synergistically associated with nitrative/oxidative stress but the mechanisms are not completely understood (Peng, Oo et al. ; Yu, Xu et al. ; Gao, Kotzbauer et al. 2008). With this in mind, the second half of my project was to determine structural and functional differences between nitrated and non-nitrated αSyn. Because αSyn is easily influenced by the environment, I first investigated structural differences of αSyn amyloid oligomers with nitrated αSyn and found using Western blot and AFM analyses that there are differences in conformations as shown by the differences in electrophoretic migrations rates on acrylamide gels and structural differences directly observed in the AFM images. Knowing that nitration causes covalent interactions, I challenged the nitrated αSyn species with β-sheet disrupters acetonitrile and HFIP. I observed that the nitrated αSyn species had increased resistance to such β-sheet disrupting treatments, suggesting the existence of inter-molecular covalent bonding. These results confirm prior literature stating nitrated αSyn demonstrates structural differences from αSyn oligomers resulting from covalent interactions (Souza, Giasson et al. 2000; Takahashi, Yamashita et al. 2002; Ruf, Lutz et al. 2008).

I also found functional differences between αSyn oligomers and the nitrated species, such as the nitrated species causing no immediate increase in intracellular Ca^{2+}, shortened LTP expression, and had no effect on pCREB levels, whereas αSyn oligomers increased intracellular Ca^{2+}, abolished LTP expression and decreased pCREB. However, the nitrated species did decrease cell viability after twenty-four hours treatment, suggesting that their toxicity may be exerted through a mechanism that does

not immediately involve membrane destabilization and abolishment of LTP expression. One possible explanation is that the nitration efficiency of the samples is not complete, and the non-nitrated αSyn portion in the sample could be seeded by the nitrated portion to form trace amounts of exceptionally toxic oligomers. Going with the idea of inter-seeding of the nitrated samples, it is probable that the time frame used in the present experiments was not long enough to observe effects on CREB dephosphorylation since this event is downstream of oligomer-induced decreased LTP expression. Currently, it cannot be excluded that pCREB might reduce in time in response to the treatment with nitrated αSyn species which may contain trace concentration of potentially seeded αSyn oligomers. Further studies should be done after isolation of nitrated αSyn from potentially non-nitrated αSyn using affinity chromatography to ensure purity of the samples.

The idea of nitrated αSyn influencing non-nitrated αSyn structure and function was first derived by studies demonstrating nitrated αSyn seeding αSyn resulting in stable oligomers and inhibition of fibril formation (Zhou, Long et al. ; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). To continue the investigation of the impact of nitrative modification in αSyn pathology, experiments using nitrated αSyn to seed αSyn (Nseeded) at a (1:10) stoichiometric ratio and stirred for five days were done to compare structural and functional differences of the seeded species with non-seeded αSyn. Western blot and AFM analyses revealed Nseeded results in oligomeric species but not fibrils, confirming prior studies showing that fibrillation is inhibited in Nseeded (Yamin, Uversky et al. 2003).

Cell viability studies revealed Nseeded is more toxic than αSyn oligomers. Indeed, investigations testing concentrations ranging from 0.5 to 4 µM demonstrates toxicity between 1 and 2 µM Nseeded being similar to that elicited by 4 µM non-seeded αSyn oligomers. Similar results were obtained in Ca^{2+} imaging experiments. These

concentration ranges from both the cell viability and Ca^{2+} imaging experiments suggest Nseeded αSyn having greater toxicity than non-seeded αSyn oligomers. Moreover, these observations provide a molecular link between synucleinopathies and oxidative/nitrative stress.

My project will impact future studies in a two-pronged approach. The pathological mechanisms involving CaN could be an effective target for pharmacological inhibition of neuronal damage brought about by aggregating αSyn in human diseases. By halting the downstream signaling caused by increased intracellular Ca^{2+} at the point of CaN inhibition could rescue synaptic dysfunction and cognitive impairment. In addition, the evidence of seeding by nitrated αSyn of exceptionally toxic αSyn oligomers is compelling because oxidative/nitrative stress has been consistently described as part of the multi-faceted mechanisms contributing to synucleinopathic human diseases. The evidence provided by my novel observations suggests a scenario whereby an individual with no CNS synuclein pathology but exposed to increased oxidative stress such as due to an inflammatory or chemical insult, could be challenged by a detrimental cascade of nitrative modifications of normal αSyn monomers resulting in profound seeding effects on the unmodified αSyn pool potentially resulting in triggering clinical synuclein pathology. Therefore, based on the results of my doctorate dissertation presented here, I propose that these collective mechanisms should be further considered in future studies as a valid target for the development of effective treatment strategies to halt or reverse αSyn-driven neuropathology in human diseases.

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