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2016

The Toxic Interaction Of Dopamine And Alpha-Synuclein: Implications For Parkinson's Disease

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Abstract

Neurodegenerative disorders are characterized by the death of specific neuronal populations and the aggregation of particular proteins into pathological inclusions. The role of protein aggregation and the factors that govern neuronal susceptibility to disease have remained unclear. In Parkinson's disease (PD), the loss of dopamine producing neurons in the substantia nigra (SN) leads to severe motor impairments, and the protein α-synuclein is found aggregated in several brain regions including the SN. The goal of this thesis was to investigate a possible mechanism for the vulnerability of dopaminergic neurons: the interaction of α-synuclein with dopamine itself. While dopamine has been suspected to contribute to cell death in PD by causing oxidative stress, I found using a lentiviral approach that a long-term increase of dopamine levels in non-transgenic mice was insufficient to produce dopamine neuron loss. In contrast, elevating dopamine in mice expressing human α-synuclein resulted in progressive nigrostriatal degeneration and an associated locomotor deficit. These findings demonstrate that dopamine toxicity is dependent on α-synuclein. To explore a possible point of convergence of these two factors, I examined the effects of dopamine on α-synuclein aggregation in the mouse brain. This led to the novel observation that dopamine increases total steady-state levels of α-synuclein oligomers and promotes modified oligomeric conformations. α-Synuclein oligomers generated in the presence of dopamine in vitro were biochemically similar to mouse-derived species and were found to be toxic to primary neurons. Taken together, these data provide evidence that dopamine-induced α-synuclein oligomers may underlie the susceptibility of dopaminergic neurons in disease.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Neuroscience

First Advisor
Harry Ischiropoulos

Second Advisor
Robert Kalb

Keywords
Alpha-synuclein, Parkinson's disease

Subject Categories
Neuroscience and Neurobiology

THE TOXIC INTERACTION OF DOPAMINE AND ALPHA-SYNUCLEIN:

IMPLICATIONS FOR PARKINSON'S DISEASE

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A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

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ACKNOWLEDGEMENT

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Neurodegenerative disorders are characterized by the death of specific neuronal populations and the aggregation of particular proteins into pathological inclusions. The role of protein aggregation and the factors that govern neuronal susceptibility to disease have remained unclear. In Parkinson's disease (PD), the loss of dopamine producing neurons in the substantia nigra (SN) leads to severe motor impairments, and the protein α-synuclein is found aggregated in several brain regions including the SN. The goal of this thesis was to investigate a possible mechanism for the vulnerability of dopaminergic neurons: the interaction of α-synuclein with dopamine itself. While dopamine has been suspected to contribute to cell death in PD by causing oxidative stress, I found using a lentiviral approach that a long-term increase of dopamine levels in non-transgenic mice was insufficient to produce dopamine neuron loss. In contrast, elevating dopamine in mice expressing human α-synuclein resulted in progressive nigrostriatal degeneration and an associated locomotor deficit. These findings demonstrate that dopamine toxicity is dependent on α-synuclein. To explore a possible point of convergence of these two factors, I examined the effects of dopamine on α-synuclein aggregation in the mouse brain. This led to the novel observation that dopamine increases total steady-state levels

of α-synuclein oligomers and promotes modified oligomeric conformations. α-Synuclein oligomers generated in the presence of dopamine *in vitro* were biochemically similar to mouse-derived species and were found to be toxic to primary neurons. Taken together, these data provide evidence that dopamine-induced α-synuclein oligomers may underlie the susceptibility of dopaminergic neurons in disease.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Neurodegenerative diseases are clinically and pathologically distinct, and yet share many fundamental features that suggest common underlying mechanisms. Diseases such as Alzheimer's (AD), Parkinson's (PD), Huntington's (HD) and amyotrophic lateral sclerosis (ALS) are typically late-onset and progressive, leading to debilitating cognitive and/or motor deficits. Despite differences in clinical presentation and the specific neuronal populations that are lost, neurodegenerative disorders share a common pathological hallmark, the presence of proteinaceous inclusions in the central nervous system. The regional and subcellular localization of these lesions differs in each disease, as well as the major protein constituents. In AD, extracellular senile plaques consist of the peptide amyloid-β (Glenner and Wong, 1984), and intraneuronal neurofibrillary tangles contain the microtubule-associated protein tau (Lee et al., 1991). In PD and related disorders, the hallmark pathological substrates are Lewy bodies and Lewy neurites, cytoplasmic inclusions containing the presynaptic protein α-synuclein (Spillantini et al., 1997). Intranuclear aggregates in HD are comprised of the protein huntingtin (DiFiglia et al., 1997), whereas the majority of ALS patients develop TDP-43 positive inclusions (Neumann et al., 2006).

Despite extensive research efforts, the relationship of aggregation and neurodegeneration remains unclear. The neuronal populations that harbor inclusion pathology only partially overlap with those that undergo cell death, and the density of inclusions is only weakly correlated with the severity of clinical symptoms (Ross and Poirier, 2005). While it was originally thought that large intracellular deposits compromise neuronal health, more recently it has been recognized that inclusions may

serve a protective role as a sink for more toxic aggregation intermediates. Indeed, small aggregates termed oligomers have emerged as potentially a major pathogenic species in multiple neurodegenerative disorders (Kayed et al., 2003). The discovery of causative genetic mutations in families afflicted with these disorders has allowed for the development of genetic animal and cellular models, greatly advancing our understanding of the molecular mechanisms involved in neurodegeneration. In many cases, the genes linked to disease encode the characteristic proteins found aggregated within inclusions, further implicating protein aggregation in disease pathogenesis (Lill and Bertram, 2011). Continued investigation is required to uncover the complex interactions between protein aggregates and cellular machinery that lead to neuron death, and to develop new therapies to combat these devastating diseases for which there is currently no cure.

1.2 Synucleinopathies

α-Synuclein aggregation into insoluble filamentous inclusions is a central pathological feature of diseases collectively referred to as synucleinopathies, including PD, dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). In 1912, Fritz Heinrich Lewy published the first descriptions of intraneuronal proteinaceous inclusions in PD patient brains (Lewy, 1912), which were later named after him (Tretiakoff, 1919). In 1997, following the discovery of a PD-linked mutation in the gene encoding α-synuclein (Polymeropoulos et al., 1997), it was found that Lewy bodies and analogous deposits in neural projections, Lewy neurites, are robustly immunoreactive for α-synuclein even in sporadic disease (Spillantini et al., 1997). These seminal findings heralded a new era of research on the role of α-synuclein in neurodegenerative diseases.

1.2.1 α-Synuclein structure and function

α-Synuclein is a small (140 residues) highly conserved protein that is abundantly expressed in brain and in part localized to presynaptic nerve terminals (Maroteaux et al., 1988; Jakes et al., 1994; Iwai et al., 1995). The physiological functions of α-synuclein appear to involve synaptic maintenance and plasticity (George et al., 1995; Withers et al., 1997), regulation of synaptic vesicle pools (Murphy et al., 2000; Nemani et al., 2010), and SNARE complex assembly (Chandra et al., 2005; Burré et al., 2010). Transgenic mice lacking α-synuclein have normal neuronal and synaptic development, though subtle deficits in dopamine release suggest a role in synaptic vesicle recycling (Abeliovich et al., 2000; Cabin et al., 2002; Yavich et al., 2004). While the native conformation of α-synuclein is under debate (Weinreb et al., 1996; Bartels et al., 2011; Wang et al., 2011; Fauvet et al., 2012a; Gould et al., 2014), its sequence and proximity to synaptic vesicles suggest that interactions with vesicular membranes may be important for its biological activity. Specifically, the N-terminal region contains seven imperfect repeats with a consensus motif of KTKEGV, resembling the lipid binding domain of apolipoproteins (George et al., 1995; Maroteaux et al., 1988). This region mediates acquisition of α-helical structure that occurs upon α-synuclein binding to lipid membranes *in vitro* (Davidson et al., 1998). Amino acids 61-95 make up the hydrophobic NAC (non-amyloid-β component of Alzheimer's disease amyloid) domain (Uéda et al., 1993), which is necessary for aggregation (Giasson et al., 2001). The C-terminal region is highly acidic and contains known sites of protein-protein interactions, metal binding, and post-translational modifications (Uversky et al., 2001a; Woods et al., 2007; Oueslati et al., 2010). For a detailed review of α-synuclein native structure and function see Chapter 3.

1.2.2 α-Synuclein pathology in disease

Significant clinical and pathological overlap between synucleinopathies suggests they may share common underlying mechanisms driven by α-synuclein toxicity. PD and MSA are primarily movement disorders, characterized by resting tremor, muscle rigidity, bradykinesia, and postural instability (collectively termed parkinsonism) (Hoehn and Yahr, 1967). DLB is also defined by parkinsonian symptoms, as well as prominent dementia that develops within one year of motor dysfunction (McKeith et al., 2005). While PD and DLB have largely indistinguishable Lewy body pathology in brainstem nuclei, DLB more commonly involves widespread cortical Lewy bodies, which may underlie the presentation of dementia (Kosaka, 1978; Lennox et al., 1989; McKeith et al., 1996; Spillantini et al., 1997). Senile plaques characteristic of AD are usually present in the cortex in DLB (Lennox et al., 1989), and conversely AD patients often have Lewy bodies in the amygdala (Kotzbauer et al., 2001). MSA is an atypical synucleinopathy in that α-synuclein-positive inclusions are present in oligodendrocytes rather than neurons, and are termed glial cytoplasmic inclusions (GCIs) (Papp et al., 1989; Wakabayashi et al., 1998; Spillantini et al., 1998a; Tu et al., 1998). GCIs are found in diverse areas of the brain, including the neocortex, hippocampus, midbrain, brainstem, and cerebellum (Arima et al., 1992; Duda et al., 2000).

Inclusion pathology has been extensively studied both histologically and biochemically from postmortem diseased human brain. Lewy bodies are classically defined as eosinophilic structures with a dense core surrounded by a diffuse halo; however, while this description is suitable for brainstem inclusions, cortical Lewy bodies have a less distinct appearance (Pollanen et al., 1993; Forno, 1996). α-Synuclein, but

not the related β-synuclein, has been identified as an integral component of Lewy bodies using a multitude of antibodies (Spillantini et al., 1997; 1998b; Takeda et al., 1998; Baba et al., 1998; Irizarry et al., 1998; Giasson et al., 2000a; Crowther et al., 2000). By electron microscopy, α-synuclein filaments from diseased brain appear unbranched, are typically 5-10 nm in width, and can reach hundreds of nm in length (Spillantini et al., 1998b). Several modifications have been identified in α-Synuclein isolated from Lewy bodies, including phosphorylation at Ser-129 and Ser-87, ubiquitination at Lys-12, Lys-21, and Lys-23, and C-terminal truncations (Fujiwara et al., 2002; Hasegawa et al., 2002; Anderson et al., 2006; Paleologou et al., 2010). In addition, tyrosine-nitrated α-synuclein is widespread in the brains of synucleinopathy patients (Giasson et al., 2000b). For a review of α-synuclein post-translational modifications *in vivo* and *in vitro*, see Chapter 3. Lewy body inclusions also contain other proteins, including neurofilaments, proteasomal subunits, and synaptophysin (Goldman et al., 1983; Schmidt et al., 1991; Ii et al., 1997; Takeda et al., 1998), as well as lipids (Gai et al., 2000).

1.2.3 α-Synuclein aggregation pathway

The fibrillar morphologies of synucleinopathy lesions have been recapitulated in numerous studies using recombinant α-synuclein *in vitro*. Like amyloids in AD and other diseases, α-synuclein is able to form amyloid-like fibrils, which are rich in β-pleated sheets and produce characteristic signals upon binding Thioflavin T and Congo Red dyes. Recombinant α-synuclein fibrils have been observed by numerous imaging and biochemical methodologies, including electron and atomic force microscopy, circular dichroism, solid-state nuclear magnetic resonance and infrared spectroscopy, and

gel electrophoresis (Conway et al., 1998; 2000a; Hashimoto et al., 1998; Giasson et al., 1999; Serpell et al., 2000; Tuttle et al., 2016). *In vitro*, the formation of fibrils follows a classical polymerization reaction, including a lag phase with little detectable fibril formation, followed by a growth phase with rapid polymerization. The rate-limiting step in α-synuclein fibrillization is thought to be the initial formation of a nucleus, likely an oligomer or small polymer of α-synuclein molecules (Wood et al., 1999; Uversky et al., 2001b). A schematic of the aggregation pathway of α-synuclein is depicted in Figure 1.1.
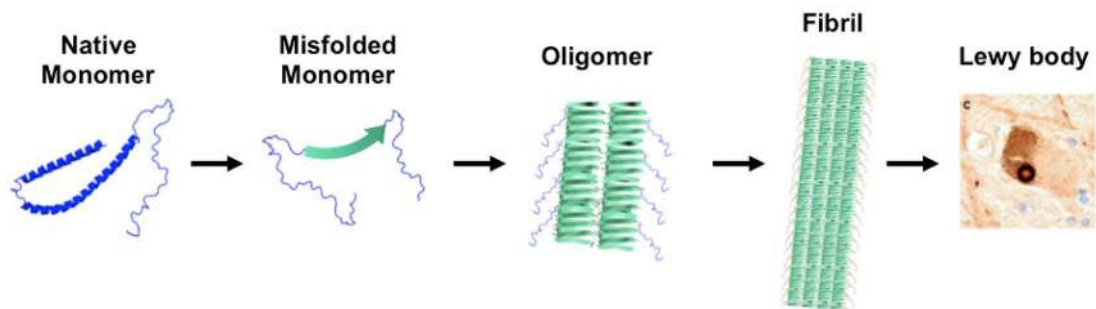
Figure 1.1: Aggregation pathway of α-synuclein. Native α-synuclein monomers must become misfolded, i.e. by genetic mutations or environmental factors, in order to be aggregation-competent. The misfolded monomer is depicted with a β-strand in the NAC domain, which participates in the formation of β-sheets. Monomers self-associate into small polymers, or oligomers, which further polymerize into highly ordered fibrils. Fibrils are then deposited into Lewy body inclusions that characterize a group of diseases known as synucleinopathies. Image of native α-synuclein monomer from the RCSB PDB (www.rcsb.org) of PDB ID 2KKW (Rao et al., 2010). Image of Lewy body from Forman et al., 2004.

1.3 Parkinson's disease

PD is the most common age-associated movement disorder, affecting 7 million people worldwide and predicted to significantly increase in prevalence as the population ages (Van Den Eeden et al., 2003; de Lau and Breteler, 2006). The parkinsonian motor features that were first described by James Parkinson in 1817 arise from progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SN) (Parkinson, 1817; Tretiakoff, 1919; Hassler, 1938). While diagnosis of PD is contingent on the presentation of motor signs, it is increasingly recognized that the PD clinical profile involves non-

motor features as well. Sleep disturbances, autonomic dysfunction, hyposmia, and depression may appear decades prior to the onset of motor impairment (Lim et al., 2009). Dementia is also common, typically in advanced PD (Reid et al., 2011). The loss of extranigral neuronal populations may underlie non-motor symptoms. In the brainstem, noradrenergic neurons of the locus coeruleus are particularly affected. Cholinergic cells are also lost, both in the dorsal motor nucleus of the vagus in the brainstem and nucleus basalis of Meynert in the basal forebrain (Forno, 1996). PD is therefore considered a complex disorder involving multiple brain regions and neurotransmitter systems.

1.3.1 Basal ganglia dysfunction in PD

The death of dopaminergic cells in PD leads to a severe depletion of dopamine at the main site of SN innervation, the dorsal striatum (Ehringer and Hornykiewicz, 1960). The SN and striatum are found within the basal ganglia, a group of subcortical structures that serve as a critical regulator of motor output. The classical model of basal ganglia circuitry describes three major pathways: the direct pathway, which is thought to promote selected motor programs, and the indirect and hyperdirect pathways, which are thought to simultaneously oppose competing motor programs (Wichmann et al., 2011). As depicted in Figure 1.2, the direct pathway involves cortical excitation of striatal neurons, which in turn disinhibit thalamocortical circuits resulting in greater excitation of motor neurons. The indirect pathway instead disinhibits the subthalamic nucleus, causing excitation of the globus pallidus interna and suppression of thalamocortical activity (Albin et al., 1989; DeLong, 1990). The hyperdirect pathway bypasses the striatum and directly excites the subthalamic nucleus, dampening the activity of motor neurons (Nambu et al., 2000).

Dopamine serves as an important modulator of this complex circuitry. Spiny projection neurons in the striatum express G-protein coupled dopamine receptors of either the D1- or D2-type. Neurons in the direct pathway express D1-type receptors, which mediate an increase in intrinsic excitability upon activation by dopamine, and promote long-term potentiation. Indirect pathway neurons express D2-type receptors and have the opposite response when bound by dopamine, promoting long-term depression. The loss of striatal dopamine in PD is therefore thought to result in reduced activity of the direct pathway and increased activity of the indirect pathway, accounting for the overall reduction of movement (Surmeier et al., 2014). While this model of basal ganglia circuitry and its dysfunction in PD has proved useful, it was recently proposed that the direct and indirect pathways may actually be much more integrated both structurally and functionally (Calabresi et al., 2014).
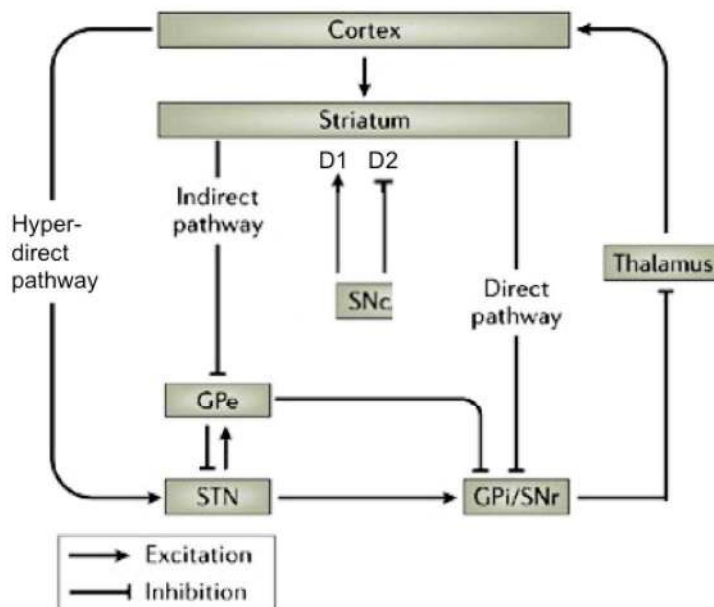
Figure 1.2. Simplified representation of basal ganglia circuitry. GPe, globus pallidus externa; GPi, globus pallidus interna; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus. Modified from Yin et al., 2006.

1.3.2 Genetics of PD

While PD is rarely inherited (~10% of all cases), genetic mutations identified in families with the disease offer insight into the mechanisms underlying the sporadic dis-order. Several genes are now linked with highly penetrant, monogenic forms of PD,

including the gene encoding α-synuclein, *SNCA*, as well as *LRRK2*, *Parkin*, *DJ-1*, *PINK1*, *UCHL1*, *ATP13A2* and *VPS35*, and numerous genetic risk factors have additionally been identified (Hernandez et al., 2016). The A53T missense mutation in *SNCA* was the first genetic link identified, specifically in autosomal dominant early-onset PD (Polymeropoulos et al., 1997). Since this discovery, additional mutations in *SNCA* have been found to cause PD: A30P, E46K, H50Q, G51D, and most recently A53E (Krüger et al., 1998; Zarranz et al., 2004; Proukakis et al., 2013; Lesage et al., 2013; Pasanen et al., 2014). These mutants are all located within the N-terminal region that is required for membrane binding, and in many cases exhibit altered aggregation kinetics and lipid interactions *in vitro* (Conway et al., 1998; Jensen et al., 1998; Giasson et al., 1999; Choi et al., 2004; Ghosh et al., 2014; Khalaf et al., 2014; Fares et al., 2014). Multiplications of the *SNCA* gene locus also cause early-onset PD, with increasing copy number correlating with earlier age of onset and greater severity of symptoms, suggesting that increased expression of wild type (WT) α-synuclein is detrimental (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ferese et al. 2015).

Mutations in *LRRK2* result in autosomal dominant PD and are the most common cause of familial cases (Zimprich et al., 2004; Paisan-Luiz et al., 2004). The G2019S mutation is the most well-studied; it is located in the kinase domain of LRRK2 and results in increased kinase activity. At present, the pathophysiological mechanisms of LRRK2 toxicity are unclear (Hernandez et al., 2016). Several of the other genes linked to PD induce a early-onset, autosomal recessive form of the disease (Kitada et al., 1998, Valente et al., 2004, Bonifati et al., 2003) and are functionally related in the cellular response to mitochondrial damage. Parkin is an E3 ubiquitin ligase that is

translocated to defective mitochondria where it is phosphorylated by PINK1. This stimulates parkin to ubiquitinate multiple substrates, leading to the clearance of mitochondria by autophagy (Park et al., 2006; Clark et al., 2006). DJ-1 may also function in this pathway, though its role is much less clear. It is thought that mutations in *Parkin*, *PINK1*, and *DJ-1* are responsible for disease through a loss-of-function mechanism (Goedert et al., 2013). Interestingly, autosomal recessive PD is not characterized by Lewy body pathology, possibly due to the young age of onset (Hernandez et al., 2016). Further investigation is necessary to better understand the role of α-synuclein in this form of PD.

1.3.3 Genetic animal models of α-synuclein toxicity

α-Synuclein expression in animal model systems has provided key insights into the complex relationship between aggregation and neurodegeneration. In monkeys and rats, viral-mediated expression of WT or A53T mutant human α-synuclein in the SN resulted in a progressive neurological disease that recapitulated the cardinal features of PD. The formation of intracellular α-synuclein positive inclusions and neuritic pathology was accompanied by the selective loss of 30-80% of dopaminergic neurons and the development of motor deficits (Lo Bianco et al., 2002; Kirik et al., 2002; 2003). Similarly, expression of A30P human α-synuclein in the rat SN caused dense α-synuclein accumulations and over 50% loss of dopamine neurons. Motor impairments were not detected in these rats, however (Klein et al., 2002), consistent with a threshold of about 70% neuron loss for movement symptoms to appear in humans (Dunnett and Bjorklund, 1999).

Efforts to model PD by transgenic overexpression of α-synuclein in mice have yielded mixed results. Expression of WT α-synuclein under the PDGF-β promoter resulted in atypical non-fibrillar α-synuclein inclusions and reduced TH immuno-reactivity in the striatum without overt dopaminergic cell loss (Masliah et al., 2000). In mice expressing human WT or familial mutants (A30P, E46K, or A53T) of α-synuclein under the PrP promoter, the SN was consistently spared of both neurodegeneration and α-synuclein inclusion pathology, despite the development of Lewy body-like inclusions in other regions specifically in A53T transgenic mice (Giasson et al., 2002; Lee et al., 2002; Gispert et al., 2003; Gomez-Isla et al., 2003; Yavich et al., 2005; Martin et al., 2006; Emmer et al., 2011). Similarly, Thy1-driven expression of human A30P α-synuclein did not result in histopathological changes or neuron loss in the SN, possibly due to low levels of transgene expression (Rathke-Hartlieb et al., 2001; Neumann et al., 2002). Targeting WT, A30P, or A53T human α-synuclein expression specifically to dopaminergic neurons using the TH promoter was insufficient to produce either nigrostriatal degeneration or Lewy body-like α-synuclein pathology (Rathke-Hartlieb et al., 2001; Matsuoka et al., 2001; Richfield et al., 2002). Greater success inducing dopaminergic cell death was achieved using bacterial artificial chromosome transgenic mice expressing human WT α-synuclein (Janezic et al., 2013) or by conditional expression of human WT or A53T α-synuclein (Nuber et al., 2008; Lin et al., 2012). (The previous paragraph is taken from Mor et al., manuscript under revision).

Invertebrates have offered simple yet powerful platforms for conducting mechanistic studies of α-synuclein toxicity. In flies, targeted expression of WT, A30P, or A53T human α-synuclein in the nervous system produces filamentous α-synuclein

inclusions, neurodegeneration of dopamine cells, and an impairment in climbing ability (Feany et al., 2000). Co-expression of α-synuclein with the chaperone Hsp70 was found to rescue neuron death without reducing inclusion pathology, suggesting that the presence of inclusions can be uncoupled from toxicity. Furthermore, co-expression of a dominant-negative mutant of Hsp70 with α-synuclein resulted in accelerated degeneration, emphasizing the role of chaperone activity in maintaining neuronal health (Auluck et al., 2002). In another study, expressing non-fibrillogenic α-synuclein lacking residues 71-82 was non-toxic to dopamine neurons, unlike WT α-synuclein, while aggregation-prone truncation mutants exacerbated toxicity (Periquet et al., 2007). *C. elegans* expressing human α-synuclein in dopaminergic neurons have exhibited variable phenotypes. In some cases, WT or A53T α-synuclein transgenic worms showed dopamine neuron loss (Lakso et al., 2003; Cao et al., 2010), whereas in another study, neurite degeneration without overt cell loss was reported in WT, A30P, and A53T worms (Kuwahara et al. 2006). Motor deficits were sometimes observed and could be rescued by administration of dopamine (Kuwahara et al. 2006; Cao et al., 2010). Finally, there is some evidence for α-synuclein accumulations in these worms (Lakso et al., 2003; Kuwahara et al. 2006); however it is unclear whether these structures are the result of α-synuclein aggregation.

1.3.4 Prion-like spread of α-synuclein pathology in PD

The progressive buildup of α-synuclein pathology in PD is suggestive of the spread of disease agent(s), potentially originating in the gut and ultimately reaching the central nervous system. Evidence suggests that early in the disease course, α-synuclein positive inclusions form in the enteric nervous system (Braak et al., 2006). The

vagal nerve serves as a major connection between the brain and the periphery, and consistent with cell-to-cell transmission of pathology, the dorsal motor nucleus of the vagus is among the earliest brain regions to become affected in PD (Braak et al., 2003). Braak and colleages have defined six stages of PD based on the stereotypical distribution of Lewy pathology. In the initial stages, in addition to the dorsal motor nucleus, the anterior olfactory nucleus develops α-synuclein positive inclusions, as well as the raphe nuclei and locus coeruleus. As PD advances, the midbrain becomes involved and finally cortical regions such as sensory association areas (Braak et al., 2003). The development of Lewy pathology may account for disease symptoms, in that inclusions are usually present in the SN by the time motor dysfunction has manifested, and neocortical lesions occur in late stages often accompanied by dementia (Goedert et al., 2013).

Several lines of evidence support prion-like transmission of α-synuclein in PD and other neurodegenerative diseases (see Table 1). Post-mortem studies of PD patients who received fetal mesencephalic grafts into the striatum showed that 11-16 years following transplantation, grafted neurons contained Lewy body-like α-synuclein inclusions (Li et al., 2008; Kordower et al., 2008). Given the young age of the grafted cells, it is unlikely that pathology developed independent of exposure to α-synuclein and/or other factors from diseased host tissue. In animal models, intracerebal inoculation of recombinant α-synuclein pre-formed fibrils (PFFs), brain tissue from symptomatic A53T α-synuclein transgenic mice, or brain tissue from synucleinopathy patients results in widespread deposition of Lewy body-like α-synuclein inclusions often accompanied by neurodegeneration and motor deficits. Moreover, it appears that, regardless of the site of injection, aggregation propagates along synaptic connections and requires the

presence of endogenous α-synuclein (Luk et al., 2012a; 2012b; Masuda-Suzukake et al 2014; Watts et al., 2013; Recasens et al., 2013; Paumier et al., 2015; Sacino et al., 2014b; Peelaerts et al., 2015). Several studies have also demonstrated the spread of α-synuclein pathology from peripheral sites to the central nervous system, using intravenous (Peelaerts et al., 2015), intramuscular (Sacino et al., 2014a), or intraperitoneal (Breid et al., 2016) injections of recombinant α-synuclein aggregates into rodents. α-Synuclein aggregation *in vitro* is also prion-like, exhibiting self-templated replication or 'seeding' via the repeated conversion of soluble protein into misfolded conformations (Conway et al., 1998; Wood et al., 1999).

While these studies provide compelling evidence that aggregated α-synuclein is sufficient to induce neurological disease bearing the hallmarks of synucleinopathies, even in otherwise healthy non-transgenic animals, many questions still remain. The injection of α-synuclein PFFs into mouse brain does not always lead to the spreading of pathology (Sacino et al., 2013; 2014b), indicating that host-dependent susceptibility must be further explored. Also, the induction of pathology may not require that the initiating α-synuclein agent be fibrillized, as recombinant monomeric α-synuclein and mutant α-synuclein lacking amino acids 71-82 in the NAC domain were able to produce inclusions in some recipient animals (Sacino et al., 2013; 2014a; Paumier et al., 2015). Finally, it is possible that factors other than α-synuclein contribute to inclusion formation, since spinal cord tissue from non-transgenic mice or mice lacking endogenous α-synuclein was able to induce pathology following intracerebral injection (Sacino et al., 2016).

Table 1.1: Major evidence for prion-like transmission of α-synuclein (agent) in cell culture and animal models (host).

Agent	Host	Findings	Reference
Recombinant human α-synuclein oligomers and PFFs of two distinct strains (fibrils or ribbons)	NonTg and human α-synuclein expressing rats, injected into SN	By 4 mpi, only ribbons induced abundant α-synuclein aggregation. Nigrostriatal degeneration was dependent on α-synuclein expression and was most severe for fibrils. Fibrils also induced motor impairment.	Peelaerts et al., 2015
	NonTg rats, injected intravenously	By 4 mpi, α-synuclein positive inclusions detected in spinal cord and cortex.	
Recombinant human and mouse α-synuclein PFFs	Non-symptomatic A53T, WT human α-synuclein Tg mice injected into hindlimb muscle	By 2-4 mpi, A53T Tg mice developed wide-spread α-synuclein aggregation in the central nervous system and motor deficits. By 12 mpi, WT Tg mice developed α-synuclein pathology without motor deficit.	Sacino et al., 2014a
PD tissue from SN containing insoluble LBs	Macaque monkeys, injected into SN or striatum	By 9 mpi, loss of striatal terminals. By 14 mpi, dopamine neuron death and diffuse α-synuclein deposits in SN.	Recasens et al., 2013
	NonTg mice, injected into SN	By 4 mpi, loss of striatal terminals, dopamine neuron death in SN, diffuse accumulations containing phosphorylated α-synuclein in SN.	
MSA basal ganglia tissue containing insoluble GCIs	Non-symptomatic A53T human α-synuclein Tg mice, injected into cortex	By ~3-4 mpi, neurological disease onset and widespread aggregates containing phosphory-lated α-synuclein.	Watts et al., 2013
Brain tissue from symptomatic A53T human α-synuclein Tg mice	Non-symptomatic A53T human α-synuclein Tg mice, injected into cortex	By ~6-7 mpi, neurological disease onset and widespread aggregates containing phosphory-lated α-synuclein.	
DLB brain tissue containing insol-uble α-synuclein	NonTg mice, injected into SN	By 15 mpi, widespread aggregates containing phosphorylated α-synuclein	Masuda-Suzukake et al., 2013
Recombinant human and mouse α-synuclein PFFs	NonTg mice, injected into SN	By 15 mpi, widespread aggregates containing phosphorylated α-synuclein, without accompanying neuron loss or motor deficit	
Recombinant human α-synuclein PFFs of two distinct strains (A or B)	Mutant tau Tg mice, injected into hippocampus	At 3, 6, and 9 mpi, strain B more efficiently cross-seeded tau than strain A.	Guo et al., 2013
	Primary neurons from NonTg or mutant tau Tg mice	Strain A was toxic, seeded abundant α-synuclein aggregation without cross-seeding tau. Strain B was non-toxic, cross-seeded tau, seeded α-synuclein less efficiently than A.	
Recombinant mouse α-synuclein PFFs	NonTg mice, injected into striatum	By 1 mpi, widespread endogenous α-synuclein aggregation. By 3 and 6 mpi, further spread of aggregation, neuron death in SN, and motor deficits.	Luk et al., 2012b

Brain tissue from symptomatic A53T human α-synuclein Tg mice	Non-symptomatic A53T human α-synuclein Tg mice, injected into cortex or striatum	Accelerated neurological disease onset, widespread aggregates containing phosphory-lated α-synuclein, reduced survival	Luk et al., 2012a
Recombinant human α-synuclein PFFs	Non-symptomatic A53T human α-synuclein Tg mice, injected into cortex or striatum	Accelerated neurological disease onset, widespread aggregates containing phosphory-lated α-synuclein, reduced survival	
WT human α-synuclein Tg mice	Mouse fetal mid-brain neurons, inj-ected into striatum	By 6 mpi, human α-synuclein from Tg mice detected in grafted cells	Hansen et al., 2011
Recombinant α-synuclein PFFs, oligomers, or monomers	WT human α-synuclein Tg mice, injected into cortex	By 1 day post-injection with PFFs or oligo-mers, intracellular puncta of α-synuclein. Diffuse α-synuclein 1 dpi with monomers.	
HEK or SH-SY5Y cultures expressing tagged α-synuclein, conditioned media	HEK or SH-SY5Y cultures expressing different tag on α-synuclein	Up to 14 days in culture, cells containing double-labeled α-synuclein were detected	
Recombinant α-synuclein PFFs, oligomers, or monomers	HEK cultures	By 3 hours post-treatment, exogenous α-synuclein was detected in cells regardless of aggregation state administered	
Recombinant human α-synuclein PFFs	Primary neurons from NonTg mice	By 14 days post-treatment, exogenous α-synuclein was internalized by cells, seeded aggregation of endogenous α-synuclein, and induced synaptic dysfunction and cell death	Volpicelli-Daley et al., 2011
WT human α-synuclein Tg mice	Mouse neuronal stem cells, injected into hippocampus	Up to 1 mpi, human α-synuclein detected in non-fibrillar aggregates in grafted stem cells, and markers of apoptosis	Desplats et al., 2009
SH-SY5Y cultures expressing human α-synuclein, or conditioned media	Primary neurons or neuronal stem cells from mice or rats, or SH-SY5Y cells	Up to 3 days in culture, detected intracellular aggregates containing exogenous α-synuclein, and markers of apoptosis	
Recombinant human α-synuclein PFFs	QBI-HEK, HeLa, and SH-SY5Y cultures expressing human α-synuclein	By 1 day post-treatment, exogenous α-synuclein seeded aggregation of endogenous α-synuclein	Luk et al., 2009

Abbreviations: DLB, Dementia with Lewy bodies; GCI, glial cytoplasmic inclusion; LB, Lewy body; mpi, months post-injection; MSA, multiple system atrophy; NonTg, non-transgenic; PD, Parkinson's disease; PFF, pre-formed fibrils; SN, substantia nigra; Tg, transgenic; WT, wild type.

1.3.5 Pharmacologic therapies

Efforts to treat PD patients have centered on restoring dopaminergic signaling in the striatum. Administration of the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), was the first treatment developed for PD (Birkmayer and Hornykiewicz, 1961) and remains the most effective therapy to date. However, prolonged use of L-DOPA can have significant side effects, such as motor fluctuations and dyskinesia (Olanow, 2015). Other dopamine replacement therapies include dopamine receptor agonists and inhibitors of dopamine metabolism (Kakkar et al., 2015). While these therapeutic strategies can provide symptomatic relief, there is currently no cure for the relentless loss of dopaminergic neurons in PD.

1.4 Dopamine as a neurotoxic molecule

Despite the clinical benefit of augmenting dopamine levels in PD patients, it has long been suspected that dopamine may in fact contribute to the development of disease (Fahn and Cohen, 1992; Jenner and Olanow, 1996; Stokes et al., 1999). Dopamine can have many fates within the synapse, including release into the synaptic cleft, reuptake into the presynaptic terminal for recycling, degradation by enzyme-catalyzed oxidation, non-enzymatic auto-oxidation, and deposition into neuromelanin, the pigmented structure for which the SN is named. The enzyme- or metal-catalyzed oxidation of dopamine can generate cytotoxic quinones, hydrogen peroxide, and oxyradicals (Graham 1978), raising important questions as to the role of dopamine in the degeneration of dopaminergic neurons.

1.4.1 Dopamine synthesis and metabolism

Dopamine is synthesized in the cytosol from the amino acid L-tyrosine. The first step in this reaction is rate-limiting; the enzyme tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to L-DOPA using molecular oxygen and tetrahydrobiopterin as cofactors. L-DOPA is converted to dopamine by aromatic amino acid decarboxylase (AADC), generating carbon dioxide as a byproduct. The end-product, dopamine, serves to regulate its own levels through feedback inhibition of TH (Nagatsu et al., 1964; Levitt et al., 1965). Two residues, Arg-37 and Arg-38, in the N-terminal regulatory domain of TH are required for this inhibition. Mutation of these residues to Glu-37, Glu-38 significantly increases the catalytic activity of TH (Nakashima et al., 2002), which was exploited in the current work to achieve unregulated elevation of dopamine levels.

Upon synthesis, dopamine is immediately packaged into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2) (Erickson et al., 1992). VMAT2 is coupled to a vesicular ATPase that generates a proton gradient, resulting in the pH within synaptic vesicles of ~5.6 (Johnson and Scarpa, 1976; Casey et al., 1977). The acidic environment facilitates neurotransmitter packaging, as two protons are exchanged for each molecule of dopamine (Knoth et al., 1981), and also prevents dopamine from becoming oxidized. Upon stimulation by an action potential, synaptic vesicles loaded with neurotransmitter fuse with the presynaptic membrane and dopamine is released into the synaptic cleft. Dopamine can then bind to D1- or D2-type postsynaptic receptors, as well as D2-type presynaptic autoreceptors, and get recycled back into the presynaptic neuron by the dopamine transporter (DAT) (Segura-Aguilar et al., 2014; Surmeier et al., 2014; Ford, 2014).

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Dopamine degradation is achieved by enzyme-catalyzed oxidation, which necessarily produces potentially toxic aldehydes and peroxides. Both in the nerve terminal and extracellularly, monoamine oxidase (MAO) catalyzes the oxidative deamination of dopamine into 3,4-dihydroxyphenylacetaldehyde (DOPAL), generating hydrogen peroxide as a byproduct. DOPAL, a recognized neurotoxin (Burke et al., 2003; Goldstein et al., 2013), is further oxidized to the non-toxic 3,4-dihydroxyphenylacetate (DOPAC) by aldehyde dehydrogenase. Extracellularly, DOPAC is converted to homovanillic acid (HVA) by catechol-*O*-methyl transferase (COMT). COMT can also act directly on dopamine to generate 3-methoxytyramine, which can in turn be metabolized to HVA by extracellular MAO and aldehyde dehydrogenase (Segura-Aguilar et al., 2014).

1.4.2 Dopamine auto-oxidation and antioxidant defenses

Dopamine that is not degraded or packaged into synaptic vesicles is able to auto-oxidize in the cytosol, generating electron-deficient quinones and reactive oxygen species (Graham 1978; Smythies et al., 1998; Sulzer et al., 2000). Dopamine contains a catechol moiety (vicinal hydroxyls on an aromatic ring) which can readily donate two electrons to form dopamine-*o*-
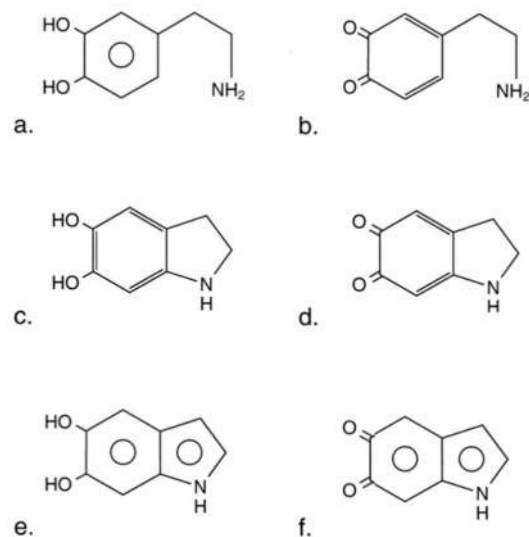
Figure 1.3.   Auto-oxidation of dopamine.
a. dopamine,   b. dopamine-*o*-quinone,
c. leukoaminochrome,   d. aminochrome,
e. 5,6-dihydroxyindole,   f. 5,6-indolequinone.
Modified from Smythies et al.,1998.

quinone (see Figure 1.3). If the electron acceptor is molecular oxygen, this generates superoxide anion radicals. Dopamine-*o*-quinone can undergo deprotonation of the amine group and cyclization to generate leukoaminochrome, which can further oxidize to form the quinone, aminochrome. The rearrangement of aminochrome to 5,6-dihydroxyindole is followed by oxidation to 5,6-indolequinone and polymerization to form neuromelanin. Neuromelanin is an insoluble, granular structure that is thought to be a protective mechanism for the sequestration of reactive derivatives of oxidized dopamine (Sulzer et al., 2000). However, the observation that pigmented midbrain neurons are preferentially lost in PD (Hirsch et al., 1988) suggests that neuromelanin and/or its precursors could directly contribute to neuronal injury.

In addition to being deposited in neuromelanin, the products of dopamine oxidation can be cleared by antioxidant enzymes and oxidant scavengers. Superoxide radicals are converted to hydrogen peroxide and molecular oxygen by superoxide dismutases (SODs). SODs are metalloenzymes localized to the cytoplasm (copper- and zinc-containing SOD) and mitochondrial matrix (manganese-containing SOD) (McCord and Fridovich, 1969). Glutathione peroxidase removes hydrogen peroxide by oxidizing glutathione, and reduced glutathione is restored by glutathione reductase. Alternatively, hydrogen peroxide can be broken down to molecular oxygen and water by catalase (Fahn and Cohen, 1992). There is some evidence that dopamine quinones may be removed via conjugation to glutathione, either spontaneously or catalyzed by glutathione *S*-transferases (Baez et al., 1997). Conjugation of dopamine-*o*-quinone to glutathione forms 5-*S*-glutathionyl dopamine, which is degraded to 5-*S*-cysteinyldopamine. The latter product has been detected in human   basal ganglia (Rosengren et al., 1985).

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1.4.3 The role of dopamine in oxidative stress

Due to the propensity of dopamine to auto-oxidize and its normal clearance via oxidative metabolism, neurons in the SN are thought to be at high risk of oxidative stress. Oxidative stress occurs when the concentration of oxidants exceeds the antioxidant buffering capacity of the cell. Oxidants such as dopamine quinones, hydrogen peroxide, and oxyradicals can damage all major classes of macromolecules, potentially leading to cellular dysfunction and death (Fahn and Cohen, 1992; Jenner and Olanow, 1996; Stokes et al., 1999). Several studies have identified markers of oxidative stress in postmortem PD brain tissue. In the SN, polyunsaturated fatty acid content was found to be reduced compared with other brain regions and tissue from controls, while products of lipid peroxidation including malondialdehyde as well as 4-hydroxynonanol protein adducts were increased (Dexter et al., 1989a; Yoritaka et al., 1996). Lewy bodies exhibit robust immunoreactivity for protein nitration, including specifically nitrated α-synuclein (Good et al., 1998; Giasson et al., 2000), and oxidatively modified nucleic acids are present in SN neurons as well (Zhang et al., 1999).

The SN contains high concentrations of iron (Hallgren and Sourander, 1958), putting this region at risk for both iron-catalyzed dopamine oxidation and production of highly toxic hydroxyl radicals. Ferric iron (Fe^{3+}) can catalyze the formation of dopamine quinones and, once it is reduced to ferrous iron (Fe^{2+}), can react with hydrogen peroxide via the Fenton reaction to produce hydroxyl radical. In the presence of superoxide radical, Fe^{2+} can be continually regenerated from Fe^{3+} in a dangerous cycle of Haber-Weiss type reactions (Fahn and Cohen, 1992). PD patients have increased total iron and specifically Fe^{3+} in SN relative to healthy controls, whereas glutathione levels are

decreased (Sofic et al., 1988; Riederer et al., 1989; Dexter et al., 1989b; 1991). These alterations are consistent with increased potential for iron-catalyzed oxidation reactions, coupled with a reduced capacity of the antioxidant response.

Dopamine quinones are potentially neurotoxic via the covalent adduction to cysteine sulfhydryl groups. Cysteines are often located in the active site of proteins, and adduction may therefore irreversibly impair protein function (Sulzer et al., 2000). Catecholamine cysteinyl adducts are selectively increased in the SN of PD patients, though it is unknown if this is due to L-DOPA therapy (Spencer et al., 1998). The protein targets of dopamine modification have been explored in various model systems (Segura-Aguilar et al., 2014). Proteomic analysis was performed on isolated rat brain mitochondria and SH-SY5Y cells that were exposed to radiolabeled dopamine or dopamine quinone. A subset of mitochondrial proteins as well as the PD-linked proteins DJ-1 and UCH-L1 were found to be covalently modified by dopamine (Van Laar et al., 2009). This is consistent with evidence that dopamine quinone alters mitochondrial function and induces swelling, effects which are inhibited by the addition of glutathione (Berman and Hastings, 1999). PD-associated parkin was found to be modified by catechols in human SN, and modification of parkin by dopamine in neuronal cell lines caused its functional inactivation (LaVoie et al., 2005). Dopamine quinone has also been reported to covalently modify and inhibit the function of TH (Xu et al., 1998; Kuhn et al., 1999) and DAT (Berman et al., 1996; Whitehead et al., 2001).

Toxin-based models of PD lend support for oxidative stress playing a role in dopaminergic cell death. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism via its active metabolite, 1-methyl-4-phenylpyridinium

(MPP[+]) (Dauer and Przedborski, 2003), an effect which was first observed in drug users who inadvertently synthesized MPTP (Davis et al., 1979; Langston et al., 1983). Postmortem studies in these individuals as well as MPTP administration in monkeys and mice has revealed nigrostriatal degeneration and depletion of striatal dopamine, consistent with PD. Lewy body pathology has not been consistently observed, however (Davis et al., 1979; Forno et al., 1993; Langston et al., 1999; Moratalla et al., 1992; Muthane et al., 1994; Seniuk et al., 1990; Varastet et al., 1994). MPP[+] is selectively taken up by dopaminergic neurons through DAT (Javitch et al., 1985), and acts to inhibit mitochondrial complex I activity (Nicklas et al., 1987). This can lead to production of superoxide and other reactive oxygen species, and consequently oxidative stress (Hasegawa et al., 1990; 1997). The insecticide rotenone also causes dopamine neuron loss by inhibiting complex I (Betarbet et al., 2000), and the herbicide paraquat, which is structurally related to MPP[+], increases levels of superoxide (Day et al., 1999). Both rotenone and paraquat are able to initiate formation of Lewy body-like α-synuclein inclusions in rodents (Dauer and Przedborski, 2003). Furthermore, there is evidence for increased risk of PD with herbicide/pesticide exposure (Tanner, 1992).

1.4.4 Animal models of dopamine toxicity

A limited number of studies have investigated whether dopamine is toxic *in vivo*. Intrastriatal injection of high concentrations of dopamine (up to 1 μmol) in rats resulted in loss of dopaminergic nerve terminals and formation of protein cysteinyl-dopamine adducts within one week. The size of the lesion correlated with the concentration of dopamine injected, and co-administration of glutathione or ascorbate both mitigated

toxicity and decreased adduct formation (Filloux and Townsend, 1993; Hastings et al., 1996; Rabinovic et al., 2000). Similarly, rats injected with aminochrome into the SN showed evidence of neurodegeneration and motor impairment (Díaz-Véliz et al., 2002; Touchette et al., 2016).

In addition to direct administration of dopamine or its oxidized derivatives, pharmacological and genetic approaches have been taken to manipulate endogenous dopamine handling and assess the effects on the nigrostriatal system. While a complete knockout of VMAT2 is lethal (Wang et al., 1997), transgenic mice expressing 5% of normal VMAT2 levels in order to increase cytosolic dopamine did not have SN cell loss (Colebrooke et al., 2006). In a different strain of the same mice, a mild, progressive degenerative phenotype was reported in combination with elevated levels of cysteinyl-catechols (Caudle et al., 2007). Amphetamines are highly toxic to dopaminergic nerve terminals, which is thought to be mediated at least in part by the redistribution of dopamine from synaptic vesicles into the cytosol (Sulzer et al., 1995; LaVoie and Hastings, 1999). Mice deficient in VMAT2 exhibit greater sensitivity to meth-amphetamine, consistent with exacerbated toxicity of cytosolic dopamine (Fumagalli et al., 1999; Guillot et al., 2008). Conversely, overexpression of VMAT2 is protective against methamphetamine-induced damage (Lohr et al., 2015). Reserpine, another inhibitor of vesicular dopamine storage, is not neurotoxic, possibly since its effects are transient (Tieu, 2011). The elevation of cytosolic dopamine by overexpression of DAT in SN neurons (Masoud et al., 2015) or ectopic DAT expression in striatal neurons (Chen et al., 2008a) has resulted in neurodegeneration and oxidative stress. Collectively, these studies emphasize the potential role of dopamine in PD etiology.

1.5 The interaction of dopamine and α-synuclein

1.5.1 Structural characterization

In 2001, Lansbury and colleagues conducted a screen for compounds that inhibit α-synuclein fibrillization *in vitro*. Of the 15 candidates identified as potent modifiers of aggregation, 14 were catecholamines. Dopamine was found to kinetically arrest soluble α-synuclein oligomers, and this effect was blocked by antioxidants (Conway et al., 2001). Subsequent studies have confirmed that dopamine stabilizes oligomer species *in vitro* even at substoichiometric concentrations, and oxidation of dopamine but not α-synuclein is required (Li et al., 2004; 2005a; Norris et al., 2005; Cappai et al., 2005; Follmer et al., 2007; Herrera et al., 2008; Pham et al., 2009; Bisaglia et al., 2010; Mazzulli et al., 2016). Consistent with these findings, elevating dopamine levels in SH-SY5Y cells expressing A53T α-synuclein reduced the number of cells containing insoluble aggregates and increased levels of soluble, high molecular weight oligomers. Moreover, the appearance of these species was prevented by antioxidant treatment (Mazzulli et al., 2006; 2007).

The requirement of dopamine oxidation suggests that α-synuclein may interact with dopamine quinones. Indeed, aminochrome was found to effectively inhibit α-synuclein fibril formation (Li et al., 2004; Norris et al., 2005), and incubation of dopamine with α-synuclein results in quinone-associated protein (Li et al., 2005a; Mazzulli et al., 2016). Interestingly, α-synuclein does not contain cysteine residues and therefore cannot form cysteinyl adducts. Mutating other reactive residues that could become covalently modified by dopamine does not have an effect, suggesting that the interaction is non-covalent. Consistent with this, dopamine-mediated inhibition of α-synuclein fibrillization is reversible (Norris et al., 2005), and does not generate

appreciable dopamine-protein adduct as measured by mass spectrometry and radiolabeling (Li et al., 2004; Norris et al., 2005; Pham et al., 2009; Bisaglia et al., 2010). The sites of interaction between dopamine and α-synuclein likely include residue E83 and the 125-YEMPS-129 motif in the C-terminus, since mutation or deletion of these sites restores the ability of α-synuclein to fibrillize in the presence of dopamine (Norris et al., 2005; Herrera et al., 2008). The dopamine-mediated increase in oligomeric species observed in SH-SY5Y cells is also prevented by expression of α-synuclein lacking the 125-YEMPS-129 domain (Mazzulli et al., 2007).

Dopamine-stabilized α-synuclein oligomers have been extensively characterized using a combination of biochemical and imaging techniques. Recombinant oligomers generated in the presence of dopamine appear spherical or dot-like by electron microscopy and atomic force microscopy, and both N- and C-termini of α-synuclein are exposed (Cappai et al., 2005; Li et al., 2005a; Norris et al., 2005; Follmer et al., 2007). These species migrate as high molecular weight bands on SDS-PAGE, indicating that they are SDS- and heat-stable. Further analysis by size exclusion chromatography reveals a range of elution times, corresponding to heterogeneous oligomer conformations (Conway et al., 2001; Cappai et al., 2005; Li et al., 2005a; Pham et al., 2009; Bisaglia et al., 2010; Mazzulli et al., 2016). By circular dichroism, dopamine-incubated α-synuclein is largely composed of random coil structure, consistent with 'off-pathway' species that do not progress to form β-sheet rich fibrils (Li et al., 2004; Cappai et al., 2005; Norris et al., 2005; Pham et al., 2009; Bisaglia et al., 2010). Collectively, these studies indicate that oxidized dopamine potently modifies α-synuclein aggregation, resulting in diverse soluble oligomeric conformers.

1.5.2 Functional characterization

Growing evidence suggests that α-synuclein oligomers are neurotoxic species. Viral expression of artificial α-synuclein variants with enhanced oligomerization in the rat SN resulted in the selective loss of dopaminergic neurons (Winner et al., 2011). Similarly, artificial mutants with impaired β-sheet structure and increased propensity to form oligomers caused degeneration of dopaminergic nerve terminals when expressed in worms and overt dopaminergic cell loss in flies (Karpinar et al., 2009). In another study, oligomer extracts from A53T α-synuclein transgenic mice were found to be toxic when applied to primary cortical cultures, while extracts immunodepleted of α-synuclein were not (Tsika et al., 2010). Recombinant α-synuclein oligomers generated *in vitro* have also been shown to be neurotoxic in culture (Danzer et al., 2007), and oligomer formation is accelerated by the A53T and A30P familial PD mutations (Conway et al., 2000b). Moreover, soluble high molecular weight oligomers have been extracted from human brain tissue and are increased in PD patients (Sharon et al., 2003).

The toxicity of dopamine-induced α-synuclein oligomers is largely unexplored. Evidence from neuronal cultures and cell-free assays suggests that these species are able to inhibit their own degradation as well as that of other substrates by chaperone-mediated autophagy (Martinez-Vicente et al., 2008). While there is no evidence that increasing dopamine in SH-SY5Y cultures produces toxic α-synuclein oligomers (Mazzulli et al., 2006; Yamakawa et al., 2010), exogenous application of recombinant dopamine-stabilized oligomers has been reported to reduce viability and neurotransmitter release in PC12 cells (Li et al., 2005a; Choi et al., 2013). A30P α-synuclein oligomers generated in the presence of dopamine reduced neurite number and length in primary

mesencephalic and cortical cultures (Follmer et al., 2007). In addition, the toxicity of endogenous cytosolic dopamine in cultured fetal human dopaminergic neurons was linked to the accumulation of soluble α-synuclein protein complexes (Xu et al., 2002).

1.6 Rationale and objectives

A major stumbling block in the development of effective treatments for PD has been the inadequate understanding of two defining disease features: the progressive death of dopaminergic neurons in the SN, and the widespread formation of α-synuclein inclusions. A longstanding hypothesis suggests that dopamine contributes to disease pathogenesis through the generation of cytotoxic quinones and other electrophiles (Graham, 1978), yet dopamine toxicity has not been extensively studied in animal models. Oxidized dopamine has been shown to stabilize potentially toxic α-synuclein oligomers in cultured cells and cell-free systems (Conway et al., 2001; Norris et al., 2005; Mazzulli et al., 2006; 2007). However, it remains unknown if dopamine is able to modify α-synuclein oligomerization *in vivo* and whether the resultant species are capable of driving neurodegeneration.

To explore the possibility that dopamine and α-synuclein interact *in vivo*, we manipulated both endogenous dopamine levels and α-synuclein expression in mice (Chapter 2). Using A53T human α-synuclein transgenic mice (Giasson et al., 2002) and a novel lentiviral approach to enhance dopamine synthesis, we aimed to accomplish three major goals: (1) to test if sustained elevation of dopamine levels induces nigrostriatal degeneration, (2) to determine if dopamine toxicity is dependent on α-synuclein expression, and (3) to test if dopamine modifies α-synuclein aggregation *in vivo*

resulting in potentially toxic species. We found that both elevated dopamine levels and α-synuclein expression are required for neurodegeneration of the SN, and that dopamine promotes conformationally and functionally modified α-synuclein oligomer species *in vivo*. In addition, we found that dopamine-stabilized recombinant α-synuclein oligomers are structurally similar to mouse-derived oligomers and are toxic to primary neuronal cultures. These findings reveal a synergistic interaction between dopamine and α-synuclein that may account for the vulnerability of dopaminergic neurons in disease.

In Chapter 3, we review what is known regarding α-synuclein physiological functions and native conformations. A model for the transition from native α-synuclein conformers to aggregation-competent species is proposed.

Finally, Chapter 4 provides a summary of our findings, their significance within the broader scientific understanding, and clinical implications. Important questions that remain following this work, as well as future studies that may address these questions, are highlighted.

CHAPTER 2

DOPAMINE-INDUCED NIGROSTRIATAL DEGENERATION IS MEDIATED BY SOLUBLE α-SYNUCLEIN OLIGOMERS

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2.1 Abstract

Parkinson's disease is defined by the loss of dopamine producing neurons in the substantia nigra (SN) and an abundance of Lewy body inclusions comprised of aggregated α-synuclein. To date, efforts to explain the selective vulnerability of dopamine neurons have been hindered by the lack of overt dopaminergic cell death in transgenic mice overexpressing α-synuclein. To address this issue, dopamine levels were manipulated in addition to α-synuclein expression, since the oxidative properties of dopamine have long been suspected to contribute to dopaminergic cell death. SN-targeted expression of mutant tyrosine hydroxylase, which is insensitive to feedback inhibition by dopamine, increased the steady-state levels of dopamine without damaging dopaminergic cells in non-transgenic mice. In contrast, raising dopamine levels in mice expressing human A53T mutant α-synuclein induced progressive nigrostriatal degeneration and a reduction of locomotion. A possible mechanism for this converging action is dopamine-mediated stabilization of α-synuclein oligomers, which occurs in cell-free and culture systems but has not been tested *in vivo*. Elevation of dopamine in A53T transgenic mice increased the total levels of soluble α-synuclein oligomers and resulted in conformationally and functionally modified species. Moreover, dopamine-induced α-synuclein oligomers generated *in vitro* decreased viability in primary neuronal cultures. The data demonstrate that SN degeneration is dependent on both dopamine levels and α-synuclein, suggesting a unique interaction by which two cardinal features of Parkinson's disease, dopaminergic cell death and α-synuclein aggregation, are linked to account for the vulnerability of this neuronal population.

2.2 Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder that is principally defined by the motor symptoms of resting tremor, rigidity, and bradykinesia. These symptoms occur primarily as a result of the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SN), which depletes dopamine at nigrostriatal synapses (Parkinson, 1817; Hassler, 1938; Ehringer and Hornykiewicz, 1960). A longstanding hypothesis to explain the vulnerability of this cell population states that dopamine induces neuronal injury via oxidative stress. The enzyme- or metal-catalyzed oxidation of dopamine generates electron-deficient quinones and reactive oxygen species that can induce cellular dysfunction (Graham, 1978; Fahn and Cohen, 1992; Jenner and Olanow, 1996; Stokes et al., 1999). The SN in PD patients is reported to harbor oxidatively modified proteins, lipids, and nucleic acids (Dexter et al., 1989a; Yoritaka et al., 1996; Zhang et al., 1999; Giasson et al., 2000). Efforts to study the effects of dysregulated dopamine in animal models have included ectopic dopamine administration or increased cytosolic dopamine by suppression of VMAT2 expression (Hastings et al., 1996; Colebrooke et al., 2006; Caudle et al., 2007).

More recently, α-synuclein has emerged as a critical player in PD pathogenesis. Disturbances in α-synuclein protein folding or expression level, i.e. through mutations or extra copies of the α-synuclein gene, are known causes of PD (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Zarranz et al., 2004; Proukakis et al., 2013; Lesage et al., 2013; Pasanen et al., 2014; Ferese et al. 2015). In both familial and sporadic cases, aggregated α-synuclein is present within Lewy bodies and Lewy neurites, which are the hallmark pathological lesions of the

disease (Spillantini et al., 1997; Baba et al., 1998). α-Synuclein is a small highly conserved protein (140 residues) that is abundantly expressed in the nervous system primarily at presynaptic terminals (Maroteaux et al., 1988; Jakes et al., 1994; Iwai et al., 1995). The precise physiological functions of α-synuclein remain unclear, though it is thought to play diverse roles in synaptic maintenance and plasticity, neurotransmitter release and homeostasis, and the regulation of synaptic vesicle pools (George et al., 1995; Withers et al., 1997; Abeliovich et al., 2000; Murphy et al., 2000; Cabin et al., 2002; Yavich et al., 2004; Chandra et al., 2005; Burré et al., 2010; Nemani et al., 2010). In disease, α-synuclein assembles into β-sheet rich amyloid-like fibrils, generating several intermediate oligomeric species of unknown toxicity (Ross and Poirier, 2005).

Intriguingly, α-synuclein oligomers are kinetically stabilized by oxidized dopamine and other catecholamines *in vitro* (Conway et al., 2001; Norris et al., 2005), providing a possible link between dopamine- and α-synuclein-mediated toxicities. Extensive evidence from cultured cells and cell-free systems indicates that dopamine potently and substoichiometrically inhibits the fibrillization of α-synuclein, resulting in the kinetic arrest of soluble oligomer species. Dopamine oxidation is required for oligomer stabilization, suggesting α-synuclein interacts with dopamine-quinone and/or other oxidation products. The interaction is non-covalent, reversible, and dependent on residues E83 and 125-YEMPS-129 motif in the C-terminus of α-synuclein (Conway et al., 2001; Norris et al., 2005; Cappai et al., 2005; Mazzulli et al., 2006; 2007; Follmer et al., 2007; Herrera et al., 2008). The toxicity of these oligomers is largely unexplored, though *in vitro* dopamine-incubated α-synuclein is reported to interfere with and resist degradation by chaperone-mediated autophagy (Martinez-Vicente et al.,

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2008), and to inhibit SNARE complex formation and neurotransmitter release (Choi et al., 2013). Importantly, it is unknown if dopamine is able to modify α-synuclein oligomerization *in vivo* and whether the resultant species are capable of driving neurodegeneration. These are the critical questions we aimed to answer in this study.

2.3 Results

2.3.1 Expression of TH-RREE elevates dopamine levels and causes hyperactivity in NonTg mice

To increase dopamine levels *in vivo*, we used a lentiviral vector carrying the cDNA of human tyrosine hydroxylase (TH). TH is the rate-limiting enzyme in dopamine biosynthesis converting tyrosine to L-DOPA, which is then metabolized by aromatic amino acid decarboxylase to generate dopamine. A mutated TH enzyme that has Arg37-Arg38 replaced by Glu37-Glu38 (TH-RREE) is insensitive to feedback inhibition by dopamine and was used to achieve increased catalytic activity of TH (Nakashima et al., 2002; Mazzulli et al., 2006; 2007). The function of the vector was first tested on human neuroblastoma SH-SY5Y cells. This cell line has undetectable levels of endogenous TH, despite the expression of other enzymes required for synthesis and processing of dopamine. The cells were transduced after 5 days of differentiation with 20 μM retinoic acid and TH expression was confirmed by western blot and immunocytochemistry compared with empty vector (CtrlVect)-transduced controls (Figure 2.S1a-c). The TH-RREE transduced cells had measurable intracellular levels of L-DOPA, dopamine, and DOPAC in the range of 25-200 fm/μg, whereas catecholamines were not detected in CtrlVect-transduced cells (Figure 2.S1d).

We performed bilateral injections of TH-RREE vector into the SN of aged (10 month old) NonTg mice. This age was selected because the mice expressing A53T mutant human α-synuclein are asymptomatic and do not exhibit the well-known motor phenotype derived from spinal cord degeneration (Giasson et al., 2002). The typical age of onset of this phenotype in our colony is approximately 18 months. At 5 months post-injection (mpi), TH expression was increased in both SN and striatum in NonTg TH-RREE mice compared to age-matched NonTg CtrlVect mice (Figure 2.1a-b).
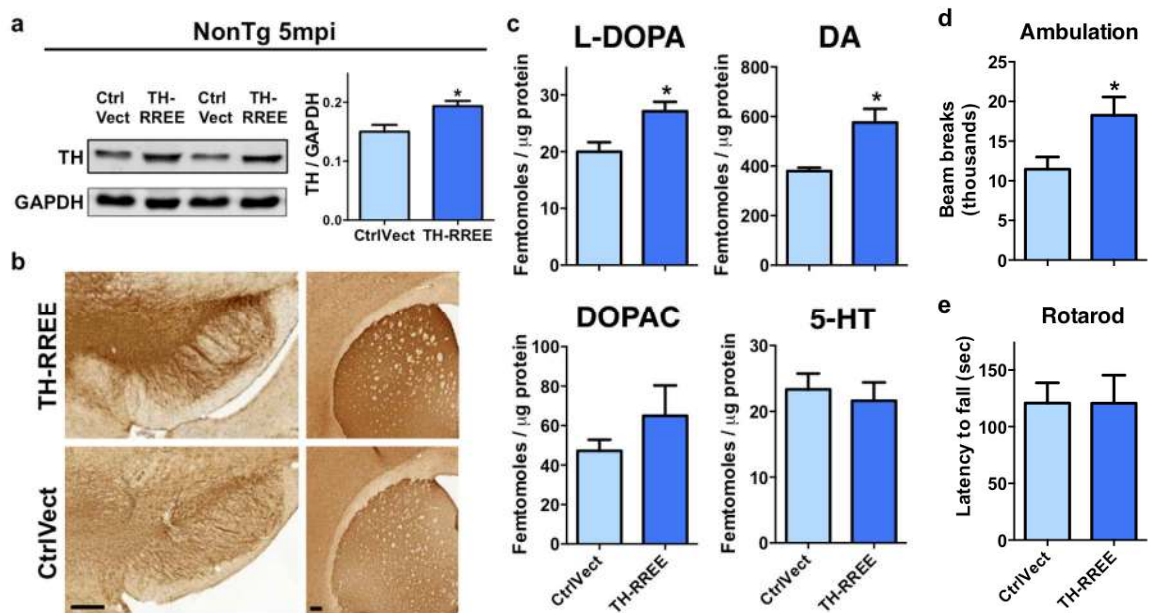
Figure 2.1: Increased dopamine levels in the striatum of NonTg mice results in hyperactivity. **(a)** TH protein levels were increased in the striatum of TH-RREE vector injected mice relative to empty vector-injected controls (CtrlVect) at 5 mpi. Densitometric analysis was conducted by normalizing TH to the GAPDH loading control. The data are presented as mean ± s.e.m. ($n = 3$; two-tailed unpaired Student's t test). **(b)** Increased TH expression was confirmed by immunohistochemistry in the SN (left panels) and striatum (right panels). Scale bar, 200 μm. **(c)** TH overexpression significantly increased the steady state concentrations of striatal catecholamines L-DOPA and dopamine (DA), but did not alter DOPAC or 5-HT. The data are presented as mean ± s.e.m. ($n = 4$ except DOPAC $n = 5$ for CtrlVect, and 5-HT $n = 3$ for TH-RREE; two-tailed unpaired Student's t test). **(d)** TH-RREE injected NonTg mice exhibited greater locomotion as measured by open field activity. The data are presented as mean ± s.e.m. ($n = 5$; two-tailed unpaired Student's t test). **(e)** There was no change in rotarod performance between the injection groups. The data are presented as mean ± s.e.m. ($n = 3$; two-tailed unpaired Student's t test). $*P < 0.05$.

TH-RREE significantly elevated striatal concentrations of L-DOPA and dopamine by 36 and 52%, respectively, while DOPAC was not affected. Importantly, 5-HT levels did not change, indicating a specific effect on the catecholamine neurotransmitter system (Figure 2.1c). Consistent with elevated striatal dopamine, NonTg TH-RREE mice exhibited hyperactivity in open field testing compared with NonTg CtrlVect mice (Figure 2.1d). There was no difference in rotarod performance between the NonTg injection groups (Figure 2.1e). These data indicate that the TH-RREE vector is able to significantly enhance dopamine levels fully 5 months following injection.

2.3.2 Dopamine-induced neurodegeneration and motor deficit is dependent on α-synuclein expression

Bilateral injection of the TH-RREE vector into the SN of 10 month old A53T mutant α-synuclein mice also achieved TH overexpression (Figure 2.S2). However, in contrast to NonTg mice, A53T TH-RREE mice exhibited striatal degeneration and neuronal loss in the SN at 5 mpi. To avoid the detection of vector-encoded TH, we used another marker of dopaminergic neurons, VMAT2, to evaluate neuronal loss. A53T TH-RREE mice had fewer VMAT2 positive cells in the SN compared with all other injection groups (Figure 2.2a). We quantified the loss of neurons using unbiased stereological counting of Nissl cells, which revealed a significant 25% reduction in A53T TH-RREE mice compared to controls (Figure 2.2b). The number of neurons quantified in age-matched A53T CtrlVect as well as NonTg CtrlVect and NonTg TH-RREE mice was typical for the mouse SN (Tieu et al., 2003), indicating that neurodegeneration had not occurred in these mice (Figure 2.2b).
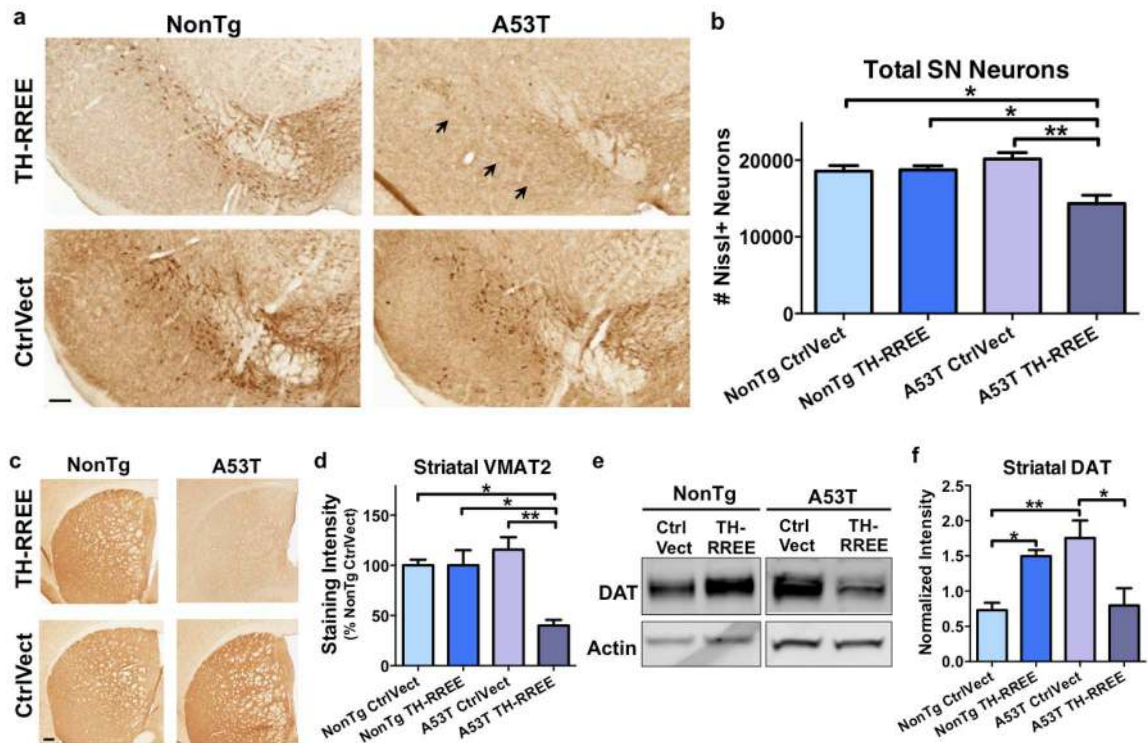
Figure 2.2: Dopamine-induced neurodegeneration of the SN is dependent on α-synuclein.
(a) At 5 mpi, fewer VMAT2 positive cells were present in the SN of A53T TH-RREE mice
(arrows) compared with all other injection groups. Scale bar, 100 μm. **(b)** Unbiased stereological
counting of Nissl-positive neurons in the SN revealed a significant 25% loss of cells in A53T TH-
RREE mice. The data are presented as mean ± s.e.m. ($n = 3$ except $n = 4$ for A53T TH-RREE;
one-way ANOVA with Tukey's correction for multiple comparisons). **(c-d)** Histological analysis
of VMAT2 staining in the striatum, with subtraction of background staining in the cortex,
indicated severe dopaminergic denervation in A53T TH-RREE mice. Scale bar, 200 μm. The
data are presented as mean ± s.e.m. ($n = 3$; one-way ANOVA with Tukey's correction for
multiple comparisons). **(e-f)** Striatal DAT levels normalized to the actin loading control were
increased in NonTg TH-RREE mice relative to NonTg CtrlVect, whereas DAT levels were
decreased in A53T TH-RREE mice as compared to A53T CtrlVect. The data are presented as
mean ± s.e.m. ($n = 3$ except $n = 4$ for NonTg CtrlVect; one-way ANOVA with Tukey's
correction for multiple comparisons). *$P < 0.05$, **$P < 0.01$.

The degeneration of cell bodies was accompanied by a significant 62% decrease

in VMAT2 staining in the striatum of A53T TH-RREE mice, demonstrating a severe loss

of dopaminergic nerve terminals (Figure 2.2c-d). Denervation of the striatum was further

corroborated by quantification of DAT levels, which revealed a significant 55% decline

in A53T TH-RREE mice compared with A53T CtrlVect. In contrast, DAT levels

were two-fold higher in NonTg TH-RREE as compared to NonTg CtrlVect mice (Figure 2.2e-f). DAT upregulation may serve to compensate for increased dopamine levels, and may not be apparent in A53T TH-RREE mice due to the substantial loss of dopaminergic terminals. The injury in A53T mice appears to be primarily presynaptic in that levels of D1 and D2 receptors remained unchanged (Figure 2.S3a-c). Levels of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32), which serves as an important signaling molecule in spiny projection neurons, were also unaffected (Figure 2.S3a,d).

To further investigate the degenerative phenotype in A53T TH-RREE mice and whether it is progressive, the total number of SN neurons was quantified at the earlier timepoint of 2.5 mpi. No difference was observed between A53T CtrlVect and TH-RREE mice, indicating that cell loss had not yet occurred (Figure 2.3a). Despite the maintenace of neuronal cell bodies, striatal VMAT2 levels revealed a 25% decrease in nerve terminal density by this timepoint, suggesting that degeneration had begun at dopaminergic synapses (Figure 2.3b). DAT levels were instead found to be increased in A53T TH-RREE mice relative to CtrlVect mice at 2.5 mpi (Figure 2.3c), similar to NonTg TH-RREE mice at 5 mpi (Figure 2.2e-f). Consistent with a compensatory increase in DAT, dopamine levels at 2.5 mpi were significantly elevated by 71% in A53T TH-RREE mice compared with age-matched A53T CtrlVect mice (Figure 2.3d).

Following the initial increase in dopamine, however, A53T TH-RREE mice subsequently exhibited a 37% drop in striatal dopamine content between 2.5 and 5 mpi. A53T CtrlVect mice showed no change in dopamine during the same time period (Figure 2.3d). The reduction in dopamine levels cannot be explained by increased conversion to DOPAC since levels of this metabolite remained unchanged from 2.5 to 5 mpi
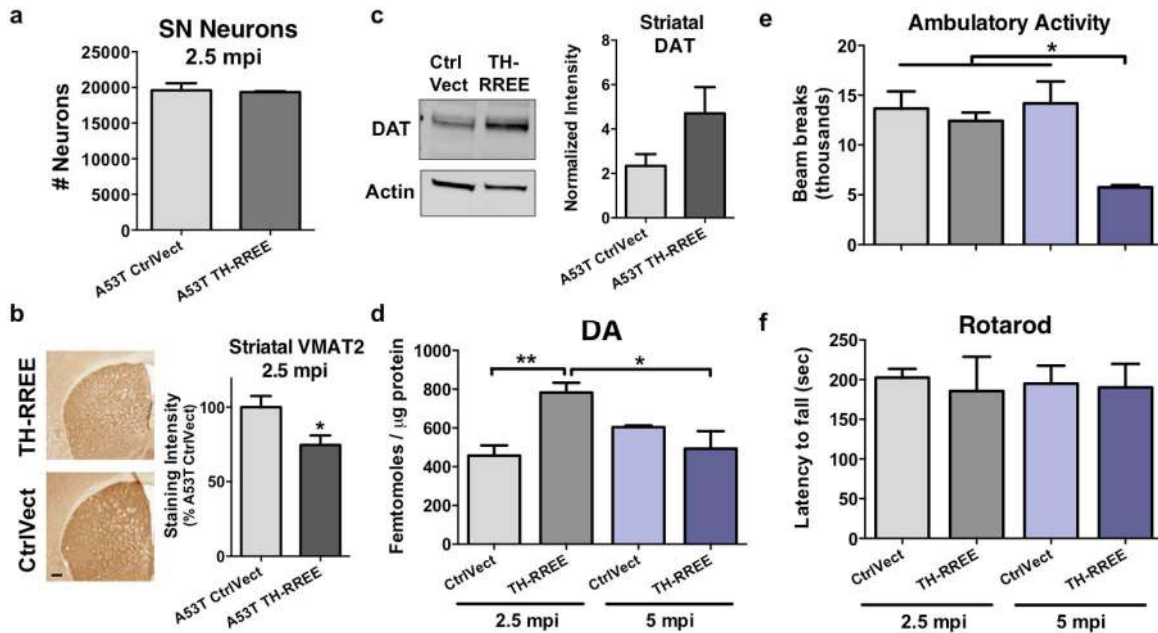
Figure 2.3: Development of the neurodegenerative phenotype in A53T TH-RREE mice ultimately leading to locomotor deficit. **(a)** At the early timepoint of 2.5 mpi, A53T TH-RREE mice did not yet exhibit loss of neuronal cell bodies in the SN. The data are presented as mean ± s.e.m. ($n = 4$ except $n = 3$ for TH-RREE; two-tailed unpaired Student's *t* test). **(b)** VMAT2 staining in the striatum revealed a modest loss of terminals suggesting that dopaminergic synapses had degenerated prior to overt cell death. Scale bar, 200 μm. The data are presented as mean ± s.e.m. ($n = 4$; two-tailed unpaired Student's *t* test). **(c)** DAT levels in the striatum of A53T TH-RREE mice were instead found to be increased at 2.5 mpi. The data are presented as mean ± s.e.m. ($n = 3$; two-tailed unpaired Student's *t* test). **(d)** A53T TH-RREE mice exhibited an elevation of dopamine (DA) levels only transiently, with an initial increase of 71% at 2.5 mpi compared with age-matched A53T CtrlVect mice. Subsequently, however, A53T TH-RREE mice underwent a significant 37% drop in striatal dopamine between 2.5 and 5 mpi, which was not observed in A53T CtrlVect mice. The data are presented as mean ± s.e.m. ($n = 3$ except $n = 5$ for TH-RREE 2.5 mpi; one-way ANOVA with Tukey's correction for multiple comparisons). **(e)** Consistent with a late-onset depletion of dopamine in the striatum, from 2.5 to 5 mpi A53T TH-RREE mice developed a reduction in locomotor activity that was not observed in CtrlVect mice. The data are presented as mean ± s.e.m. ($n = 3$; one-way ANOVA with Tukey's correction for multiple comparisons). **(f)** The motor deficit in A53T TH-RREE mice was not severe enough to affect coordination or balance, as rotarod performance remained intact. The data are presented as mean ± s.e.m. ($n = 4$ except $n = 3$ for TH-RREE 2.5 mpi and CtrlVect 5 mpi; one-way ANOVA with Tukey's correction for multiple comparisons). *$P < 0.05$, **$P < 0.01$.

(Figure 2.S4a). We also did not detect an increase in dopamine-protein adducts by near infrared fluorescence (Figure 2.S4b-c). Rather, the loss of dopamine is likely due to the ongoing nigrostriatal degeneration documented in these mice. Concomitant with the decrease in dopamine levels, A53T TH-RREE mice exhibited a significant impairment in ambulatory activity that was not apparent at 2.5 mpi but emerged by 5 mpi (Figure 2.3e). Performance on the rotarod remained intact, suggesting that balance and coordination were not disrupted (Figure 2.3f). Collectively, the results show that dopamine promotes the progressive neurodegeneration of dopaminergic neurons and a specific motor deficit in mice that express A53T α-synuclein, but not in NonTg mice.

2.3.3 Dopamine modifies α-synuclein oligomer conformations in A53T mice

The data indicate that increased steady state levels of dopamine result in neurotoxicity in an α-synuclein dependent manner. To investigate possible mechanisms, we examined the influence of dopamine on α-synuclein aggregation, and in particular the kinetic stabilization of potentially toxic α-synuclein oligomers. The presence of Lewy body-like inclusions in circumscribed regions including the brainstem of aged A53T mice has been well documented (Giasson et al., 2002). α-Synuclein-positive inclusions were indeed detected in the brainstem of A53T TH-RREE mice at 5 mpi (age 15 months), similar to age-matched A53T CtrlVect mice (Figure 2.S5a). In contrast, the SN is known to remain devoid of inclusions during the lifespan of A53T mice (Giasson et al., 2002). Expression of TH-RREE vector did not induce α-synuclein inclusion formation or alter levels of detergent-insoluble α-synuclein in the SN at 5 mpi (Figure 2.S5).

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Examination of detergent-soluble α-synuclein extracted from SN of A53T mice at 5 mpi revealed significant alterations in the quantities and conformations of α-synuclein species as a result of dopamine elevation. A53T TH-RREE mice had a significant 22% reduction in monomeric human α-synuclein compared with A53T CtrlVect mice (Figure 2.4a-b). Upon fractionation of the soluble SN extract by native size exclusion chromatography (SEC), we detected the presence of various oligomeric α-synuclein species of different molecular weights (Figure 2.4c). Quantification of total oligomeric species revealed a significant increase in A53T TH-RREE mice, greater than two-fold above A53T CtrlVect mice (Figure 2.4d). To characterize the oligomers, we used multiple α-synuclein antibodies and a combination of SDS-PAGE, immunoelectron microscopy, and biochemical assays. In A53T CtrlVect mice, oligomers had Stokes radii of up to 65 Å and ranged in molecular weight from 36 to 80 kD (Figure 2.4c and Figure 2.S6a-b), consistent with previous observation (Tsika et al., 2010). However, in addition to these low Å species, larger species of up to 122 Å were detected in A53T TH-RREE mice. On 12% SDS-PAGE, these species migrated as dimers, trimers, and high molecular weight polymers stable to SDS and heat, consistent with the presence of oxidized/crosslinked species (Souza et al., 2000). The appearance of unique, high Å species in A53T TH-RREE mice was observed with multiple α-synuclein antibodies that recognize N- or C-terminal epitopes (Figure 2.4c and Figure 2.S6a-b). Moreover, analysis of quinone-associated protein by near-infrared fluorescence revealed that only the high Å oligomeric fractions from A53T TH-RREE mice were positive, supporting a direct interaction of dopamine and α-synuclein oligomers in these mice (Figure 2.S6c).
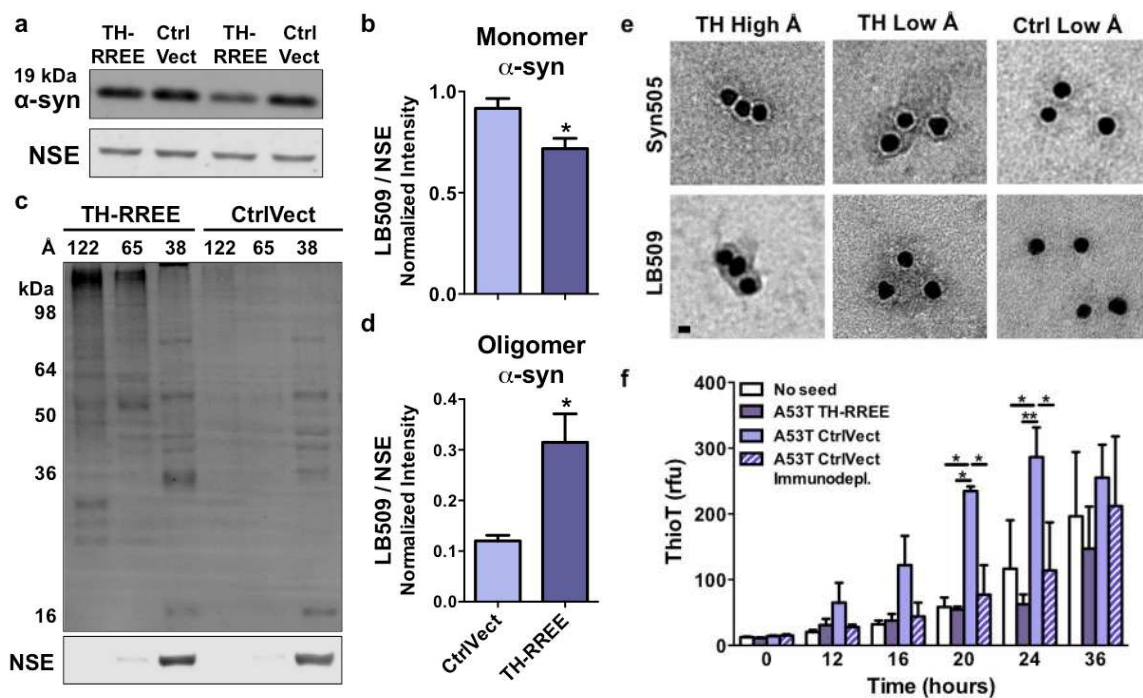
Figure 2.4: Dopamine induces conformationally distinct α-synuclein oligomers *in vivo*. (a-d) The SN of A53T TH-RREE and A53T CtrlVect mice at 5 mpi was extracted with 1% Triton and the soluble fraction was analyzed by SDS-PAGE **(a-b)**. The soluble extract was further subjected to native size exclusion chromatography and fractions corresponding to 31-38 Å, 41-65 Å, and 72-122 Å were pooled and analyzed by SDS-PAGE **(c-d)**. Neural specific enolase (NSE) was used as a loading control. The quantification revealed a decrease in soluble α-synuclein monomer and a corresponding increase in α-synuclein oligomers in A53T TH-RREE compared with A53T CtrlVect. The oligomer species in A53T TH-RREE mice were present in higher Å fractions than in controls and had a range of molecular weights. The data are presented as mean ± s.e.m. (Monomer, $n = 3$ for CtrlVect and $n = 5$ for TH-RREE; Oligomer, $n = 3$ for CtrlVect and $n = 4$ for TH-RREE; two-tailed unpaired Student's *t* test). **(e)** Immunoelectron microscopy on low (41-65) and high (72-122) Å fractions using Syn505 (N-terminal) and LB509 (C-terminal) α-synuclein antibodies, with 10 nm gold-conjugated secondary. Scale bar, 10 nm. **(f)** Pooled oligomeric fractions (41-122 Å) from indicated mice were added to aggregation reactions with fresh recombinant α-synuclein, and fibril formation was monitored by Thioflavin T (ThioT). Prior immunodepletion of α-synuclein from A53T CtrlVect fractions was performed as a control. rfu, relative fluorescence units. The data are presented as mean ± s.e.m. ($n = 3$ except $n = 4$ for no seed and immunodepl; repeated measures two-way ANOVA with Tukey's correction for multiple comparisons). *$P < 0.05$, **$P < 0.01$.

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Further structural analysis of oligomers isolated from high (72-122) or low (41-65) Å SEC fractions was done by immunoelectron microscopy. Oligomers typically appeared as clusters with >2 gold particles in both high and low Å fractions from A53T TH-RREE mice as well as low Å fractions from A53T CtrlVect mice. The species were labeled with α-synuclein antibodies directed at either the N- or C-terminus, indicating that both ends were exposed (Figure 2.4e). Clusters of >2 gold particles were not observed in high Å fractions from A53T CtrlVect mice or fractions from A53T TH-RREE mice that were immunodepleted of α-synuclein prior to imaging.

Since there is growing evidence for prion-like spreading of α-synuclein pathology in disease (Goedert et al., 2010), we investigated if dopamine promotes oligomeric species with the capacity to propagate *in vivo*. α-Synuclein oligomers extracted from A53T TH-RREE and A53T CtrlVect mice (pooled 41-122 Å SEC fractions) were monitored in a seeding assay with fresh recombinant α-synuclein *in vitro*. In the case of CtrlVect mice, oligomers efficiently seeded aggregation relative to the no-seed condition, as measured by Thioflavin T (Figure 2.4f). Importantly, this effect was abolished by prior immunodepletion of α-synuclein from the SEC fractions. In contrast, the same fractions from TH-RREE mice were unable to act as seeds (Figure 2.4f), suggesting that seeding-competent species were not retained following the interaction of α-synuclein with dopamine *in vivo*. Collectively, these data indicate that dopamine promotes the generation of conformationally and functionally modified α-synuclein oligomeric species in the mouse brain.

2.3.4 Dopamine-induced α-synuclein oligomers are neurotoxic in primary cultures

To further establish the link between dopamine-induced oligomers and toxicity, we used purified recombinant human wildtype α-synuclein co-incubated with dopamine *in vitro*. Consistent with prior reports (Conway et al., 2001; Norris et al., 2005; Cappai et al., 2005; Follmer et al., 2007), analysis of amyloid formation with Thioflavin T indicated that at equimolar concentrations, dopamine potently inhibited α-synuclein fibrillization (Figure 2.5a). In the absence of dopamine, α-synuclein adopted typical β-sheet structure indicative of amyloid formation, whereas under conditions with dopamine, α-synuclein retained random coil structure (Figure 2.5b). Sedimentation analysis confirmed that dopamine promotes soluble α-synuclein oligomer species that are SDS- and heat-stable (Figure 2.5c). Similar to mouse-derived oligomers, recombinant species ranged from 36 to >98 kD and were recognized by both N- and C-terminally directed α-synuclein antibodies in western blots and by immunoelectron microscopy (Figure 2.5c-d). Imaging by electron microscopy revealed clusters of >2 gold particles as observed in oligomer fractions from mouse SN (Figure 2.5d and Figure 2.4e). These findings suggest that dopamine-induced oligomers generated *in vitro* share common epitopes and potentially similar conformations to oligomers resulting from dopamine elevation *in vivo*.

The toxicity of recombinant oligomers was tested using primary mouse neuronal cultures. Hippocampal neurons were cultured for one week prior to treatment with the products of α-synuclein incubation with dopamine or control treatments. Two weeks post-treatment, exogenous α-synuclein from oligomer preparations had become internalized within neurons and localized to neurites (Figure 2.6a). At this timepoint, cell viability was evaluated using Calcein AM and propidium iodide (PI) dyes.

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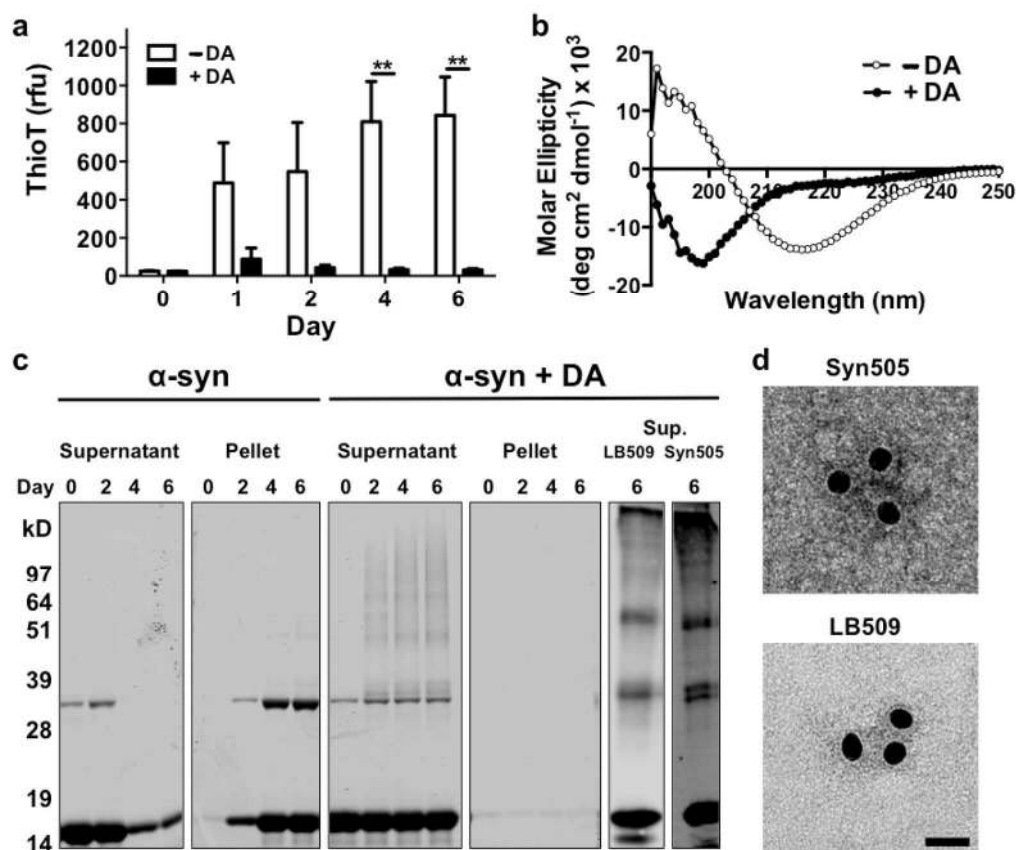
Figure 2.5: Biochemical and structural characterization of α-synuclein oligomers resulting from incubation with dopamine *in vitro*. **(a)** Incubation of recombinant wildtype human α-synuclein under standard aggregation conditions (400 μM, 37°C, 1400 rpm) resulted in Thioflavin T (ThioT)-positive fibril formation, whereas ThioT signal was abolished in the presence of equimolar dopamine (DA). rfu, relative fluorescence units. The data are presented as mean ± s.e.m. (*n* = 3; repeated measures two-way ANOVA with Bonferroni's correction for multiple comparisons). **(b)** α-Synuclein fibrils exhibited characteristic β-sheet secondary structure by circular dichroism, while α-synuclein species resulting from interaction with dopamine maintained random coil structure on day 5 of incubation. Representative traces are shown. **(c)** Sedimentation analysis and coomassie blue staining revealed that over the 6-day incubation, in the absence of dopamine, soluble α-synuclein (supernatant) was lost while insoluble α-synuclein (pellet) accumulated. In the presence of dopamine, α-synuclein did not become insoluble but instead was detected as multiple soluble oligomer species. Similar to *ex vivo* oligomers extracted from A53T TH-RREE mice, *in vitro* generated oligomers were immunoreactive to Syn505 (N-terminal) and LB509 (C-terminal) α-synuclein antibodies. **(d)** Immunoelectron microscopy on α-synuclein from day 4 of incubation with dopamine confirmed both Syn505 and LB509 labeling. Scale bar, 20 nm. **$P < 0.01$.

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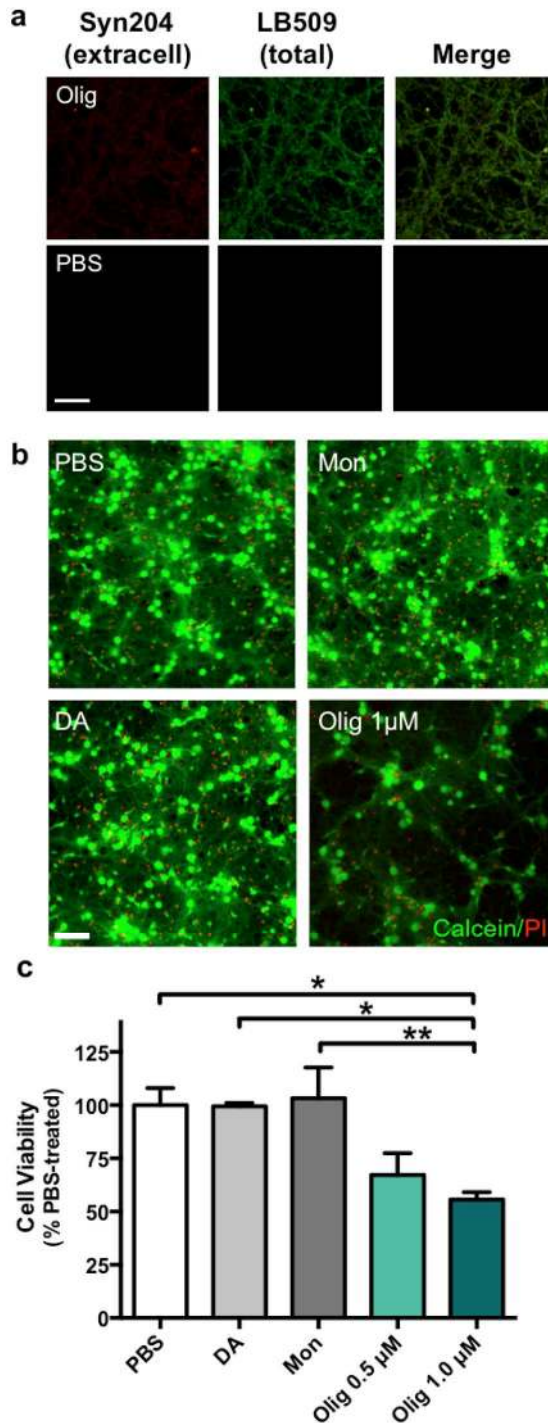
Figure 2.6: α-Synuclein oligomers generated *in vitro* in the presence of dopamine become internalized and are toxic to primary neurons. Hippocampal neurons at 7 days in vitro were exposed to 0.5 or 1 μM α-synuclein oligomer that had been prepared in the presence of dopamine (Olig), or 1 μM monomer (Mon), 1 μM dopamine that was incubated in parallel to oligomers but without α-synuclein (DA), or an equivalent dose of PBS. **(a)** Two weeks post-treatment with 1 μM Olig or PBS, cells were labeled with human α-synuclein antibodies in a two-stage protocol. Cells were live-incubated with Syn204 antibody to label extracellular α-synuclein. Following fixation and permeabilization, the cells were incubated with LB509 to label total α-synuclein. Confocal imaging showed puncta that were labeled by LB509 but not Syn204, indicating that exogenous oligomers had been internalized. These puncta were localized to neurites. Scale bar, 10 μm. (*n* = 3). **(b-c)** At two weeks post-treatments, cell viability was assayed using Calcein AM and propidium iodide (PI) dyes. The number of viable cells, defined as Calcein AM positive and PI negative, was significantly reduced by exposure to 1 μM Olig but was not affected by Mon or DA controls. Treatment with 0.5 μM Olig was insufficient to induce toxicity, indicating that the effect is dependent on dose. Scale bar, 100 μm. The data are presented as mean ± s.e.m. (*n* = 4 except *n* = 3 for PBS and DA, and *n* = 5 for 1 μM Olig; one-way ANOVA with Tukey's correction for multiple comparisons). *$P < 0.05$, **$P < 0.01$.

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Exposure of neurons to 1 μM dopamine-incubated α-synuclein induced a significant 44% reduction in Calcein positive-PI negative cells relative to PBS treated controls (Figure 2.6b-c). Treatment with equivalent doses of monomeric α-synuclein, or dopamine that had been incubated under aggregation conditions but without α-synuclein, did not reduce cell viability (Figure 2.6b-c). The observed neurotoxicity can therefore be attributed specifically to α-synuclein oligomers, consistent with our findings *in vivo* linking dopamine-modified species with severe nigrostriatal degeneration.

2.4 Discussion

In this study we used a novel lentiviral vector approach to enhance dopamine levels specifically in the SN of transgenic mice expressing the human A53T familial PD mutant of α-synuclein. In this well-characterized mouse model, the SN does not develop α-synuclein inclusions and does not undergo neuronal cell loss (Giasson et al., 2002). However, elevation of dopamine induced significant nigrostriatal degeneration and a previously undescribed ambulatory phenotype in A53T mice. Neurodegeneration of the SN was not observed in NonTg mice receiving the same treatment, indicating that dopamine-induced toxicity is dependent on α-synuclein expression. Furthermore, we demonstrate for the first time *in vivo*, dopamine-mediated changes in the total levels of α-synuclein oligomers as well as their biochemical and functional properties, suggesting that disrupting this interaction may be a promising new target in PD treatment.

Dopamine has long been considered a contributor to the death of dopaminergic neurons in disease (Fahn and Cohen, 1992; Jenner and Olanow, 1996; Stokes et al., 1999). The acidic conditions within synaptic vesicles serve to maintain

dopamine in a reduced state. However, at physiological pH in the cytosol, dopamine readily auto-oxidizes to form reactive quinone species, hydrogen peroxide, and other electrophiles (Graham, 1978). Despite the importance of understanding mechanisms of dopamine neurotoxicity, few studies have examined the role of dopamine in SN neurodegeneration *in vivo*. Intrastriatal injection of dopamine in rats resulted in protein-bound catechols and injury to dopaminergic nerve terminals after seven days. The size of the lesion was correlated with increasing concentrations of dopamine, and was diminished by co-administration with glutathione or ascorbate indicating that oxidation of dopamine was responsible for the injury (Hastings et al., 1996). Transgenic mice expressing 5% of normal VMAT2 levels had an age-dependent decline in striatal dopamine that was accompanied by decreased rotarod performance without degeneration of the SN (Colebrooke et al., 2006). In the same transgenic mice, a mild progressive nigrostriatal degeneration in aged mice was reported (Caudle et al., 2007). These studies indicate that cytosolic dopamine could be toxic, but the long-term effects of raising nigrostriatal dopamine levels has remained unknown.

The present study provides the first investigation of dopamine toxicity by chronic enhancement of dopamine synthesis *in vivo*. By expressing a mutant TH enzyme lacking residues that are critical for feedback inhibition by dopamine (Nakashima et al., 2002; Mazzulli et al., 2006; 2007), striatal dopamine in NonTg mice was increased by over 50%. Surprisingly, this significant perturbation of dopamine homeostasis was insufficient to induce SN degeneration. NonTg TH-RREE mice maintained a normal number of neurons in the SN, intact synaptic contacts with the striatum, and robust motor coordination/balance. The fate of excess dopamine in these animals is likely

release into the synapse, leading to the observed increase in locomotion. This is consistent with other studies in which increased extracellular dopamine in the striatum resulted in hyperactivity (Zhuang et al., 2001; Lohr et al., 2014). Dopamine can therefore be well-tolerated in the absence of an additional burden or defect.

α-Synuclein has been independently linked to PD through the discovery of dominantly inherited mutations and multiplications of the *SNCA* gene (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Zarranz et al., 2004; Proukakis et al., 2013; Lesage et al., 2013; Pasanen et al., 2014; Ferese et al. 2015). In non-familial PD, which accounts for roughly 90% of cases, aggregated wild type α-synuclein is present in Lewy body and Lewy neurite inclusions (Spillantini et al., 1997; Baba et al., 1998). Efforts to model PD by transgenic overexpression of α-synuclein in mice have yielded mixed results. Expression of wild type α-synuclein under the PDGF-β promoter resulted in atypical non-fibrillar α-synuclein inclusions and reduced TH immunoreactivity in the striatum without overt dopaminergic cell loss (Masliah et al., 2000). In mice expressing human wild type or familial mutants (A30P, E46K, or A53T) of α-synuclein under the PrP promoter, the SN was consistently spared of both neurodegeneration and α-synuclein inclusion pathology, despite the development of Lewy body-like inclusions in other regions specifically in A53T transgenic mice (Giasson et al., 2002; Lee et al., 2002; Gispert et al., 2003; Gomez-Isla et al., 2003; Yavich et al., 2005; Martin et al., 2006; Emmer et al., 2011.) Similarly, Thy1-driven expression of human A30P α-synuclein did not result in histopathological changes or neuron loss in the SN, possibly due to low levels of transgene expression (Rathke-Hartlieb et al., 2001; Neumann et al., 2002). Targeting wild type, A30P, or A53T

human α-synuclein expression specifically to dopaminergic neurons using the TH promoter was insufficient to produce either nigrostriatal degeneration or Lewy body-like α-synuclein pathology (Rathke-Hartlieb et al., 2001; Matsuoka et al., 2001; Richfield et al., 2002). Greater success inducing dopaminergic cell death was achieved using bacterial artificial chromosome transgenic mice expressing human wild type α-synuclein (Janezic et al., 2013) or by conditional expression of human wild type or A53T α-synuclein (Nuber et al., 2008; Lin et al., 2012). In our study, we used transgenic mice with PrP-driven expression of A53T α-synuclein (Giasson et al., 2002). In late age, these mice have a documented increase in basal dopamine levels in the striatum relative to NonTg mice (Tsika et al., 2010), which is consistent with our quantification of both catecholamines and DAT levels. However, degeneration of the SN does not occur in A53T mice without further enhancement of dopamine levels by TH-RREE expression.

In stark contrast to NonTg mice, elevation of dopamine levels in A53T mice resulted in previously undescribed, progressive nigrostriatal degeneration with a locomotor impairment, markedly improving this mouse as a model of PD. Within 2.5 months after TH-RREE vector injection into A53T mice, dopaminergic innervation to the striatum assessed by VMAT2 had decreased by 25%, while cell bodies in the SN remained intact. By 5 mpi, denervation had progressed to 62% loss of VMAT2-positive fibers with corresponding decrease in DAT, and a significant loss of neurons had occurred. These degenerative changes coincided with a reduction of dopamine levels in the striatum, and the development of a hypoactive phenotype. Notably, the initial increase in dopamine in A53T TH-RREE mice at 2.5 mpi was not accompanied by hyperactivity, as observed in NonTg TH- RREE mice at 5 mpi, consistent with an

underlying loss of terminals and dopaminergic dysfunction at this early timepoint. At 5 mpi, A53T TH-RREE mice showed significantly reduced ambulation despite dopamine concentrations similar to age-matched A53T controls. This may reflect a functional imbalance among synapses that were lost and those that remained and were overproducing dopamine (from TH-RREE expression). There was no change in the amount of dopamine receptors or DARPP-32, further supporting a presynaptic basis for the locomotor deficit.

The observed toxicity of dopamine only in A53T mice pointed to a potential synergistic role of dopamine and α-synuclein in mediating neurodegeneration. In cell-free systems, oxidized dopamine has been shown to kinetically stabilize soluble α-synuclein oligomers (Conway et al., 2001; Norris et al., 2005; Cappai et al., 2005; Follmer et al., 2007). In SH-SY5Y cultures expressing A53T α-synuclein, dopamine was able to reduce large intracellular aggregates and increase steady-state levels of soluble oligomers (Mazzulli et al., 2006; 2007). α-Synuclein oligomers are thought to be neurotoxic species. Lentiviral vector delivery of artificial α-synuclein variants with enhanced oligomerization into the rat SN resulted in the selective loss of dopaminergic cells (Winner et al., 2011). Similarly, artificial mutants with impaired β-sheet structure and increased propensity to form oligomers induced degeneration of dopaminergic nerve terminals when expressed in worms and overt dopamine cell loss in flies (Karpinar et al., 2009). However, the toxicity of dopamine-induced α-synuclein oligomers is largely unexplored. Evidence from studies conducted *in vitro* suggests that these species can block their own degradation and that of other substrates by chaperone-mediated autophagy (Martinez-Vicente et al., 2008), and may also reduce neurotransmitter

release by inhibition of SNARE complex formation (Choi et al., 2013). Moreover, the toxicity of endogenous cytosolic dopamine in cultured fetal human dopaminergic neurons was linked to accumulation of soluble α-synuclein protein complexes (Xu et al., 2002).

Critically, the effects of dopamine on α-synuclein oligomerization *in vivo* have not been investigated. We therefore sought to determine if A53T TH-RREE mice had alterations in oligomer biochemical and/or functional properties. Soluble α-synuclein oligomers were extracted from the SN and enriched by SEC under non-denaturing conditions in order to preserve native conformations. Increasing dopamine resulted in greater total levels of α-synuclein oligomers detected by western blot. The oligomers included species with Stokes radii of up to 65 Å that were also detected in A53T CtrlVect mice, consistent with previous characterization of oligomers in aged A53T mice (Tsika et al., 2010). Larger oligomers of up to 122 Å were uniquely observed in A53T TH-RREE mice, suggesting that dopamine is capable of modifying oligomer conformations. These species may reflect dopamine-induced remodeling of existing oligomers, and/or formation of oligomers from α-synuclein monomers *de novo*. Additionally, oligomers with larger Stokes radii may contain a greater number of monomer units than lower Å species, and/or occupy less compacted arrangements.

Multiple biochemical and imaging approaches were employed to further characterize the oligomers extracted from SN. In addition to the well-established methods such as western blotting and SEC, immunoelectron microscopy was performed on SEC fractions to visualize mouse-derived α-synuclein oligomers. Currently, there is no accepted method of specifically imaging α-synuclein oligomers in brain tissue *in situ*. However, with our approach, we were able to identify and image oligomers of known

Stokes radii. Oligomer species from both A53T TH-RREE and A53T CtrlVect mice were labeled with antibodies directed at the N- or C-terminus of α-synuclein, suggesting that epitopes at both ends of the protein were exposed. These epitopes were also exposed in soluble α-synuclein oligomers generated with dopamine *in vitro*, consistent with previous imaging by immunoelectron microscopy of recombinant dopamine-induced α-synuclein oligomers (Norris et al., 2005). Furthermore, LB509 and Syn505 antibodies are known to react robustly with α-synuclein in pathological inclusions in human disease brain (Jakes et al., 1999; Duda et al., 2002). The reactivity of both mouse-derived and recombinant oligomers with these antibodies suggests they may share disease-associated conformations.

Unlike the α-synuclein species extracted from A53T CtrlVect mice, oligomers from A53T TH-RREE mice were unable to act as seeds for α-synuclein fibrillization *in vitro*. These data are consistent with the known kinetic arrest of recombinant α-synuclein oligomers comprised of primarily random coil structure in the presence of dopamine (Conway et al., 2001; Norris et al., 2005). In a comparison of different oligomer species generated *in vitro*, α-synuclein oligomers that were toxic to SH-SY5Y cultures were also incapable of seeding intracellular aggregation of α-synuclein. Conversely, seeding-competent oligomers were found to be non-toxic when applied to SH-SY5Y cells (Danzer et al., 2007). These findings are in line with both our *in vivo* findings and the observed toxicity of recombinant dopamine-induced oligomers in primary neuronal cultures. Dopamine-modified species may resist sequestration into non-toxic fibrils or inclusions, and may therefore be available to participate in toxic interactions that ultimately drive neuron death.

Collectively, this work underscores the potential critical importance of the interaction of dopamine and α-synuclein in driving disease. In PD, dopaminergic terminals are thought to degenerate prior to cell bodies (Cheng et al., 2010), suggesting that the disease may arise at the synapse. Increasing dopamine levels in A53T mice recapitulated the substantial loss of terminals that precedes overt nigral cell death, offering a new model of disease progression in PD. These mice also underwent an eventual decline in dopamine levels and developed an associated motor deficit, mimicking the depletion of striatal dopamine and resulting hypokinesia that occurs in PD. Our findings also demonstrate that dopamine modifies α-synuclein aggregation *in vivo*, resulting in oligomer conformations that are biochemically and structurally similar to neurotoxic oligomers induced by dopamine *in vitro*. Dopamine-modified α-synuclein species may mediate neurodegeneration through the disruption of cellular membranes as previously proposed (Sulzer et al., 2001). Moreover, damage to synaptic vesicle membranes could lead to dopamine leakage and further induction of pathogenic α-synuclein oligomers.

2.5 Materials and Methods

Animals. The mice used in this study were homozygous for expression of human A53T α-synuclein under the mouse PrP promoter (line M83). These mice undergo spinal cord degeneration at approximately 18 months of age in our colony, and the phenotype has been described previously (Giasson et al., 2002). Any animals showing symptoms of spinal cord degeneration, i.e. hunched back, altered gait, or hindlimb paralysis, were excluded from the study. NonTg littermates were also used for experiments. No care

was taken as to the sex of the animals. Mice were randomly assigned to the lentiviral vector injection groups.

Production of Lentiviral Vectors and Determination of Titer. Generation of human tyrosine hydroxylase-1 containing the R37E/R38E mutation (TH-RREE) construct has been previously described (Mazzulli et al., 2006). The coding sequence was removed from the pcDNA3.1 vector and was subcloned into the self-inactivating pTY-linker lentiviral expression vector at the *Pme*1 site downstream of the CMV promoter. Generation of replication-deficient pseudotyped HIV-derived lentiviral vectors was achieved by transient co-transfection of HEK293T cells with the expression vector along with the vectors carrying the additional transcripts required for encapsulation, packaging and envelope proteins. 24 hours after the cells were seeded into 150 mm dishes coated with poly-D-Lysine they were transiently transfected with a mixture containing 550 μg of the pTY-TH-RREE expression vector, 357.5 μg of CMVD82 packaging vector, and 192.5 μg of pVSV-G envelope vectors using the calcium phosphate transfection protocol. The culture medium was changed every day and the media containing the virus was collected 72 hours post-transfection, filtered through 0.45 μm membranes, and concentrated by ultracentrifugation at 50,000 g for 2 hours at 4°C. The viral pellet was subsequently re-suspended in 800 μL of DMEM medium to make a concentrated viral stock. An empty vector control virus containing all elements except for the TH-RREE gene (CtrlVect) was generated in parallel and both viruses were obtained from the University of Pennsylvania Vector Core.

To determine vector functionality and viral titer, SH-SY5Y human neuroblastoma cells (American Type Culture Collection) were plated in 24-well plates at 5×10^4

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cells/well in DMEM/F12 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were allowed to recover for 2 days and were then differentiated for 5 days with 20 μM retinoic acid. The cells were infected with different dilutions of lentivirus containing either an empty vector or TH-RREE expression vector and the viral titers were calculated by TH immunocytochemistry.

Stereotaxic Injection of Lentiviral Vector into the SN. A53T α-synuclein transgenic mice or non-transgenic littermates received bilateral lentiviral vector injections at 10 months of age. The mice were placed into an anesthesia chamber connected to an isofluorane delivery system to induce anesthesia. The mice were then placed into a stereotaxic head holder, an anesthesia mask was fitted, and an incision was made in the skin. Small bilateral holes were drilled into the skull according to the coordinates for the SN in the mouse brain (anterior-posterior -3.3 mm from bregma, mediolateral 1 mm) and a 30g Hamilton syringe was inserted slowly to 4 mm dorsoventral depth. 2 μl of the viral concentrate ($5x10^7$ IU/ml) was injected into the SN at a rate of 0.25 μl/min, and the needle was left in place for an additional 5 min before being slowly withdrawn, in order to minimize leakage of the injected fluid. The skin was sutured sterilely and the animals were monitored until they recovered from anesthesia. All animal work was conducted according to National Institute of Health guide for the care and use of laboratory animals and were approved by the Children's Hospital of Philadelphia.

Motor Function Testing. All behavioral tests were conducted 1 week prior to sacrifice. Mice were habituated to the testing room for 2 hours before testing. To measure open field activity, the mice were singly housed and the cage was placed

inside a laser monitoring device consisting of an open, rectangular frame containing sensors (Opto-M3 activity meter, Columbus Instruments). The number of infrared beam breaks was quantified over a 12-hour period during the dark cycle from 6 pm to 6 am using Multi Device Interface software (Columbus Instruments). Data was collected for 3 consecutive days for each animal, and the average of the 3 days was used as $n = 1$ for each mouse. Food and water were available *ad libitum*.

To assess motor coordination and balance, mice were tested on the Rotarod (Ugo Basile, model 7650). The mice were acclimated to the apparatus with four training sessions of 5 min each at 4 rpm, followed by 5 min of rest in the home cage. Testing was performed one hour later with two trials in which speed was accelerated from 4 to 40 rpm in 300 sec. The latency to fall off the rotarod within this time period was recorded and the average of the two trials was used as $n = 1$ for each mouse.

Immunohistochemistry and Stereological Cell Counts. For histological analysis the mice were deeply anesthetized and perfused transcardially with saline followed by 4% PFA in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed overnight at 4°C in 4% PFA and then cryoprotected in 30% sucrose solution for 2 days at 4°C. The brains were submerged in dry ice-chilled isopentane for 30 sec and then stored at -80°C until sectioning. On the day of sectioning, brains were mounted on chucks with OCT (Tissue-Tek) and the brainstem, SN, and striatum were each sectioned using a cryostat (Leica, Jung Frigocut 2800N). Free-floating coronal sections of alternating 10 and 30 μm thickness were collected from each region and stored at 4°C in 0.1 M phosphate buffer containing 0.01% sodium azide.

The sections were quenched in 5% H_2O_2 in methanol for 30 min, followed by

antigen retrieval by boiling in citrate solution pH 6 for 10 min. The tissue was blocked

for 1 hour at RT with 5% NGS, 3% BSA in PBS containing 0.1% Triton X-100, and then

incubated overnight at 4°C with primary antibody diluted in blocking buffer. Antibodies

were used against VMAT2 (rabbit, 1:20000; courtesy of Dr. Gary Miller at Emory

University, Atlanta, GA), TH (rabbit, 1:2000, Calbiochem 657012), and Syn505 (mouse,

1:5000, courtesy of Dr. Virginia M. Lee at University of Pennsylvania, Philadelphia, PA).

Incubation with biotin-conjugated secondary antibodies was followed by avidin-biotin-

peroxidase complex, each for 1 hour at RT, and visualization by 3,3-diaminobenzidine

(DAB) for 1 min (ABC Elite, Vector Laboratories). In some cases, sections were

counterstained with Cresyl Violet. The tissue was then mounted, dehydrated in an

increasing alcohol series and cleared in xylenes, and coverslipped with Permount

mounting medium. Slides were scanned by the Children's Hospital of Philadelphia

Pathology Core facility. Quantification of VMAT2 staining intensity in the striatum was

performed using Image J software (National Institutes of Health).

Unbiased stereological quantification of neurons in the SN was performed as

described previously (Tieu et al., 2003). Briefly, 30 μm sections spaced 90 μm apart

were selected in order to represent the full rostro-caudal axis of the SN. The sections

were stained with Cresyl Violet and the Nissl-positive neurons were blindly counted

using the optical fractionator method (StereoInvestigator; MBF Bioscience).

Catechol Quantification by HPLC with Electrochemical Detection. Striatal

tissues were homogenized by sonication in 10 volumes of 0.1 M perchloric acid

containing 1 μM 3,4 dihydroxybenzylamine as an internal standard. The samples were

centrifuged at 16,000 g for 10 min at 4°C and the supernatants were filtered through

0.22 μm filters. Catechols were resolved on a reverse-phase C18 Luna column (150x4.6 mm, 5 μm; Phenomenex) as previously described (Mazzulli et al., 2006). Protein pellets were solubilized in 50 mM Tris pH 7.4 containing 2% SDS and protein concentration was determined using the BCA microassay kit (Pierce). Monoamine levels were normalized to protein concentration and expressed as femtomoles analyte /μg of protein.

Sequential Extraction and Native Size Exclusion Chromatography. Striatal or SN tissue from individual mice was homogenized in 10 volumes of lysis buffer: 1% Triton X-100 in 20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, and protease inhibitor cocktail (P2714, Sigma). The tissue was grinded with a mechanical homogenizer and centrifuged at 16,000 g for 10 min at 4°C. The pellet was further extracted by sonication in 2% SDS, 50 mM Tris pH 7.4 with protease inhibitor cocktail, boiling at 95°C for 10 min, and centrifugation at 16,000 g for 10 min. The resulting supernatant was designated the Triton-insoluble fraction. Protein concentration was determined using the BCA assay (Pierce).

For size exclusion chromatography (SEC), 500 μg of Triton-soluble SN tissue in a total volume of 270 μl was loaded onto a Superdex 200 HR10/30 column (GE Healthcare) connected to an Agilent 1100 series HPLC system. Mobile phase consisted of 25 mM HEPES and 150 mM NaCl, pH 7.25 and the flow rate was set to 0.3 mL/min. Fractions corresponding to 122-94, 94-81, 81-72, 72-65, 65-59, 59-54, 54-50, 50-45, 45-41, 41-38, 38-34, and 34-32 Å were each pooled and concentrated with 3,000 NMWL Ultracel Microcon filters (Millipore). Fractions 122-72, 65-41, and 38-32 were further combined to enhance the α-synuclein signal on SDS-PAGE. The SEC column was calibrated using globular protein standards (GE Healthcare).

Western Blotting. Proteins sequentially extracted or further fractionated by SEC were run on 10 or 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked for 1 hour at RT in 5% (w/v) milk in 20 mM Tris pH 7.4, 150 mM NaCl and 0.1% Tween. Incubation with primary antibodies diluted in blocking buffer was overnight at 4°C. Antibodies used against synaptic proteins were TH (rabbit, 1:4000, Calbiochem 657012), DAT (rat, 1:1000, Millipore MAB369), D1 receptor (rat, 1:1000, Sigma D2944), D2 receptor (rabbit, 1:1000, Millipore AB5084P), DARPP-32 (rabbit, 1:1000, Cell Signaling 2306S). Antibodies directed against α-synuclein were LB509 (mouse, 1:1000), Syn505 (mouse, 1:1000), and SNL-4 (rabbit, 1:1000), all courtesy of Dr. Virginia M. Lee at University of Pennsylvania, Philadelphia, PA. Loading controls for Triton-soluble proteins were GAPDH (mouse, 1:10000, Abcam ab8245), NSE (rabbit, 1:4000, Abcam ab53025), and Actin (rabbit, 1:1000, Sigma A2066). The loading control for Triton-insoluble proteins was Vimentin (mouse, 1:1000, Abcam ab8069). Membranes were incubated for 1 hour at RT with secondary antibodies conjugated to IRDye 680 or 800 (1:5000, Rockland) and then scanned using an Odyssey Infrared Imaging System (Li-Cor). Quantification of protein levels was performed using ImageStudio software (Li-Cor) and was normalized to loading control levels. For α-synuclein oligomer bands, all individual immunobands >19 kD that were detected were quantified and the intensities summed, and the total was divided by the total NSE.

Near-infrared (nIRF) scanning of oxidized catechols was performed as described previously (Mazzulli et al., 2016). Briefly, prior to transfer the SDS-PAGE gels were scanned in the 700 nm channel at intensity 10 on an Odyssey Infrared Imaging System (Li-Cor).

Immunoelectron Microscopy of SEC Fractions. SEC fractions from SN tissue corresponding to low (41-65) and high (72-122) Å Stokes radii or dopamine-incubated recombinant α-synuclein were applied to 300 mesh carbon-coated grids and blocked with 1% BSA in 20 mM Tris pH 7.4, 150 mM NaCl. The α-synuclein oligomers were labeled with the Syn505 antibody against the N-terminus or LB509 antibody against the C-terminus, followed by 10 nm gold conjugated secondary (Electron Microscopy Sciences). Grids were negatively stained with 1% uranyl acetate and imaged at the University of Pennsylvania Electron Microscopy Resource Laboratory. Control samples were immunodepleted of α-synuclein (see below) prior to grid preparation or had primary antibody omitted.

In Vitro **Aggregation and Seeding Assays.** Recombinant human wild type α-synuclein was expressed and purified as described previously (Tsika at el., 2010). Purified α-synuclein was incubated at 6 mg/mL (415 μM) with or without equimolar dopamine at 37°C and shaking at 1400 rpm for up to 6 days. At indicated time points, fibrillar content of the reaction mixture was assayed by the addition of Thioflavin T (Sigma) to a final concentration of 25 μM. Fluorescence emission was measured at 482 nm during excitation at 450 nm. Sedimentation analysis was performed by centrifugation at 16,000 g for 10 min at 4°C. The supernatants and pellets were boiled in SDS sample buffer at 95°C for 10 min. α-Synuclein aggregates were resolved by SDS-PAGE and the gels were stained with Coomassie Blue R-250. Circular dichroism spectra were obtained using a Jasco J-810 spectropolarimeter at the Children's Hospital of Philadelphia Protein Core facility. The protein was diluted to 20 μM in 0.05 M KH_2PO_4 pH 7.8. Spectra were corrected for baseline measurement of an equivalent volume of PBS diluted in

KH$_2$PO$_4$ buffer.

Seeding assays were performed by incubating 5 μg total protein from pooled oligomeric SEC fractions (41-122 Å) with 425 μg fresh recombinant α-synuclein (300 μM final concentration) at 37°C and shaking at 1400 rpm. Aliquots at indicated time points were analyzed by Thioflavin T. To immunodeplete α-synuclein for control samples, pooled SEC fractions were incubated overnight at 4°C with LB509 antibody at a 1:5 ratio of antibody to total μg protein. Protein G-conjugated beads (Sigma) were equilibrated in 25 mM HEPES, 150 mM NaCl, pH 7.4 and incubated with the immunocomplexes for 1 hour at 4°C. α-Synuclein oligomers were then pulled down by centrifugation at 2000 g for 2 min, and the resulting supernatant was used for experiments.

Primary Neuronal Cultures. Hippocampal neurons were provided by the University of Pennsylvania Neuron Culture Service Center. After dissection from C57BL/6 mouse embryos at day 18-19, cells were mechanically dissociated, trypsinized, and seeded at 100,000 cells per well into 24-well plates freshly coated with 50 μg/ml poly-D-lysine (Sigma). After plating, neurons were cultured for 2 hours in culture media containing 5% heat-inactivated fetal bovine serum, 1% Glutamax, 2% B-27 supplement, 100 U/ml penicillin, 100 μg/ml streptomycin in Neurobasal media (all from Invitrogen). After 2 hours, the media was changed to culture media without fetal bovine serum to discourage survival of glial cells. Twice per week, half of the media was replaced with fresh culture media.

Following one week in culture, neurons were treated with dopamine-incubated α-synuclein (from day 4-6 of *in vitro* aggregation) at a final concentration of 1

μM. Control conditions were equivalent doses of PBS, monomeric α-synuclein, or dopamine that had been incubated in parallel under aggregation conditions but without α-synuclein. Two weeks post-treatment, cell viability was assayed using calcein AM (Sigma) and propidium iodide (PI) (Sigma) incubated for 20 min at RT at final concentrations of 3 μM in PBS. Fluorescence images were obtained using MetaMorph software (Molecular Devices) and an inverted Olympus IX70 microscope equipped with an IX-FLA fluorescence observation attachment (Olympus Optical Co., Tokyo, Japan). For quantification of viable cells (calcein-positive and PI-negative), 10 random fields of view were blindly counted per well, with at least 100 total cells counted per replicate.

To image human α-synuclein in treated neurons, cells were labeled in a two-stage protocol as previously described (Volpicelli-Daley et al., 2011). Two weeks following treatment, cells were live-incubated in media containing Syn204 antibody (mouse IgG2a, 1:500) for 1 hour at 4°C to label extracellular α-synuclein. The cells were then fixed with 4% PFA for 20 min at RT, and permeabilized with 0.1% Triton X-100 in 5% NGS, 3% BSA in PBS for 1 hour at RT. Cells were incubated with LB509 (mouse IgG1, 1:500) overnight at 4°C to label both extracellular and intracellular α-synuclein. Secondary antibodies conjugated to Alexa Fluor (anti-mouse IgG1 488, 1:500, Thermo Fisher A21121; anti-mouse IgG2a 594, 1:500, Thermo Fisher A-21135) were incubated for 1 hour at RT. The staining was imaged by laser-scanning confocal microscopy (Olympus Fluoview).

Statistics. All statistical analysis was done using Prism 6 software (GraphPad). Two-tailed unpaired Student's *t* test was used for all comparisons between two groups. For comparisons with multiple groups, one-   way ANOVA with Tukey's correction for

multiple comparisons was used. Specifically for Thioflavin T assays with multiple groups measured over time, repeated-measures two-way ANOVA with Tukey's or Bonferroni's correction for multiple comparisons was used. All experiments were performed at least twice, with measurements from the same samples treated as technical replicates and averaged to obtain final values.

2.6 Acknowledgements

Author Contributions. DEM, ET, JRM, and HI conceived and designed the experiments. DEM, ET, JLG, and VXT performed the experiments and analyzed the data. DEM wrote the paper, with important contributions from JRM, ET, JHW, HI.

2.7 Supplementary Information
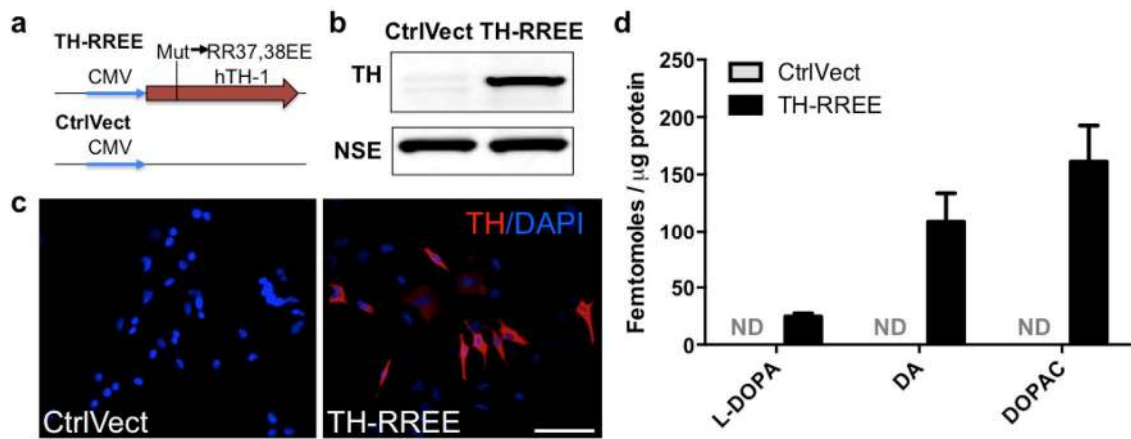
Figure 2.S1: Increased TH expression and steady-state catecholamine concentrations in SH-SY5Y cells transduced with TH-RREE vector. **(a)** Schematic representation of the lentiviral vectors used in this study. TH-RREE contained the gene for human tyrosine hydroxylase isoform 1 (hTH-1) with mutations R37E, R38E rendering TH insensitive to feedback inhibition by dopamine. The gene was absent from the empty vector control (CtrlVect), while all other elements were retained. **(b)** Western blot analysis of lysates showed TH protein only in TH-RREE transduced cells. Neural specific enolase (NSE) was used as a loading control. **(c)** TH immunofluorescence confirmed expression in TH-RREE transduced cells. Scale bar, 100 μm. **(d)** Catecholamines were detected in cells treated with TH-RREE lentivirus. ND, not detected. The data are presented as mean ± s.e.m. ($n = 3$ except $n = 6$ for DA TH-RREE and DOPAC TH-RREE).
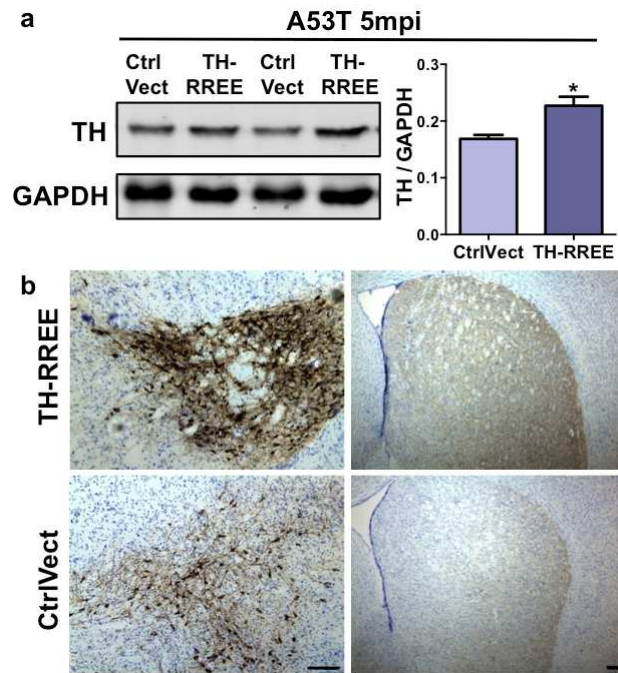
Figure 2.S2: TH-RREE vector increases TH expression in A53T mice. **(a)** TH levels were increased in the striatum in A53T TH-RREE mice compared with A53T CtrlVect at 5 mpi. GAPDH was used as a loading control. The data are presented as mean ± s.e.m. ($n = 3$; two-tailed unpaired Student's t test). **(b)** TH staining was increased in both the SN (left panels) and striatum (right panels) of A53T TH-RREE mice at 5 mpi. Cresyl Violet (Nissl) counterstain. Scale bar, 10 μm. *$P < 0.05$.
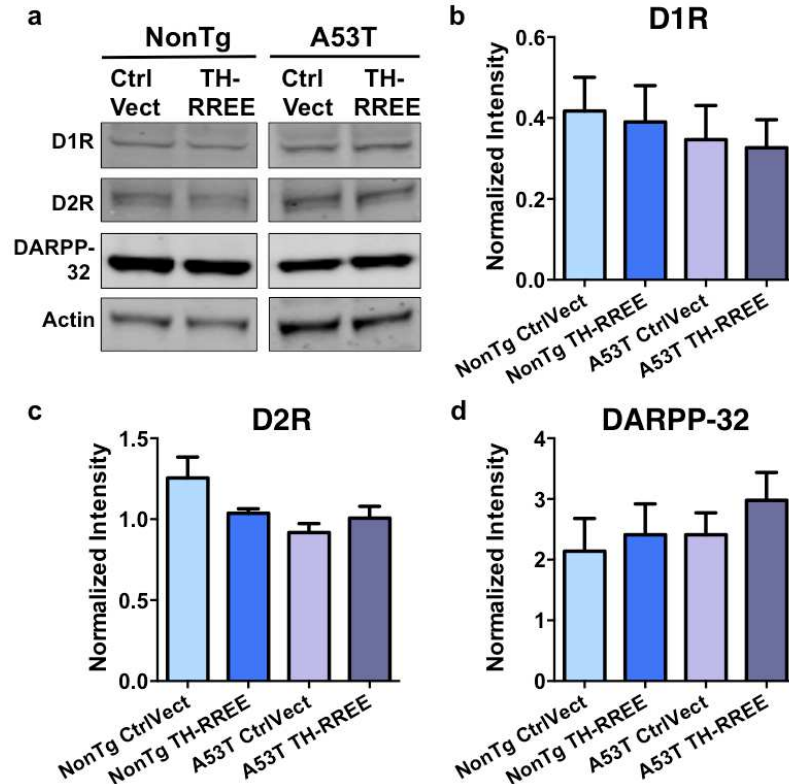
Figure 2.S3: Postsynaptic markers remain unchanged by lentiviral vector treatment. Striatal levels of D1 **(a,b)** and D2 **(a,c)** postsynaptic receptors were unaltered in TH-RREE injected A53T and NonTg mice compared with CtrlVect-injected mice. The postsynaptic signaling molecule DARPP-32 was also unaffected by viral treatment **(a,d)**. All quantification was normalized to actin loading control. The data are presented as mean ± s.e.m. (D1R, $n = 3$ except $n = 4$ for NonTg CtrlVect; D2R, $n = 3$ except $n = 4$ for NonTg CtrlVect; DARPP-32, $n = 3$; one-way ANOVA with Tukey's correction for multiple comparisons).
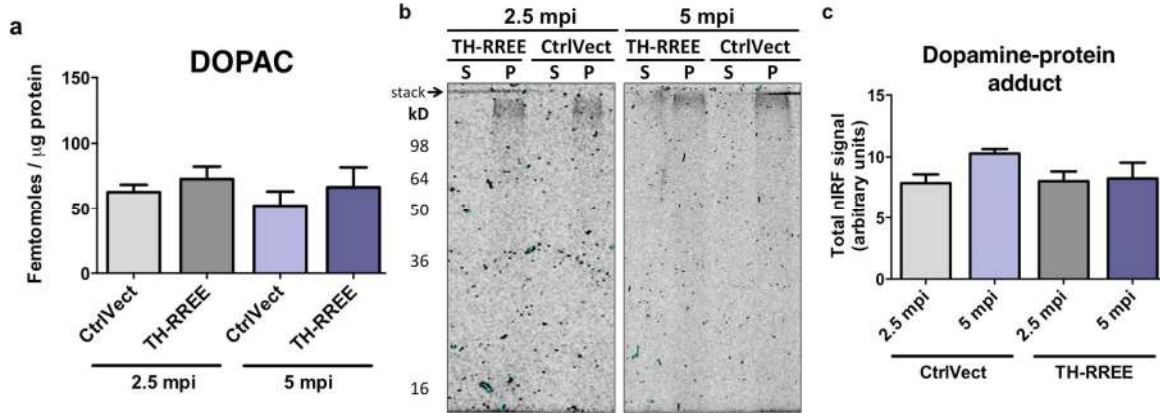
Figure 2.S4: Loss of striatal dopamine in A53T mice cannot be accounted for by metabolism to DOPAC or formation of dopamine-protein adducts. **(a)** Levels of DOPAC in the striatum remained unchanged over time regardless of lentiviral treatment. The data are presented as mean ± s.e.m. (CtrlVect, $n = 3$; TH-RREE, $n = 6$ for 2.5 mpi and $n = 4$ for 5 mpi; one-way ANOVA with Tukey's correction for multiple comparisons). **(b-c)** Triton-soluble (S) and insoluble (P) extracts of striatal tissue from mice at 2.5 or 5 mpi were analyzed by SDS-PAGE and near-infrared fluorescence (nIRF). Total levels of dopamine-protein adduct (combined soluble and insoluble nIRF signal) did not change either between injection groups or within groups over time. The data are presented as mean ± s.e.m. ($n = 3$; one-way ANOVA with Tukey's correction for multiple comparisons).
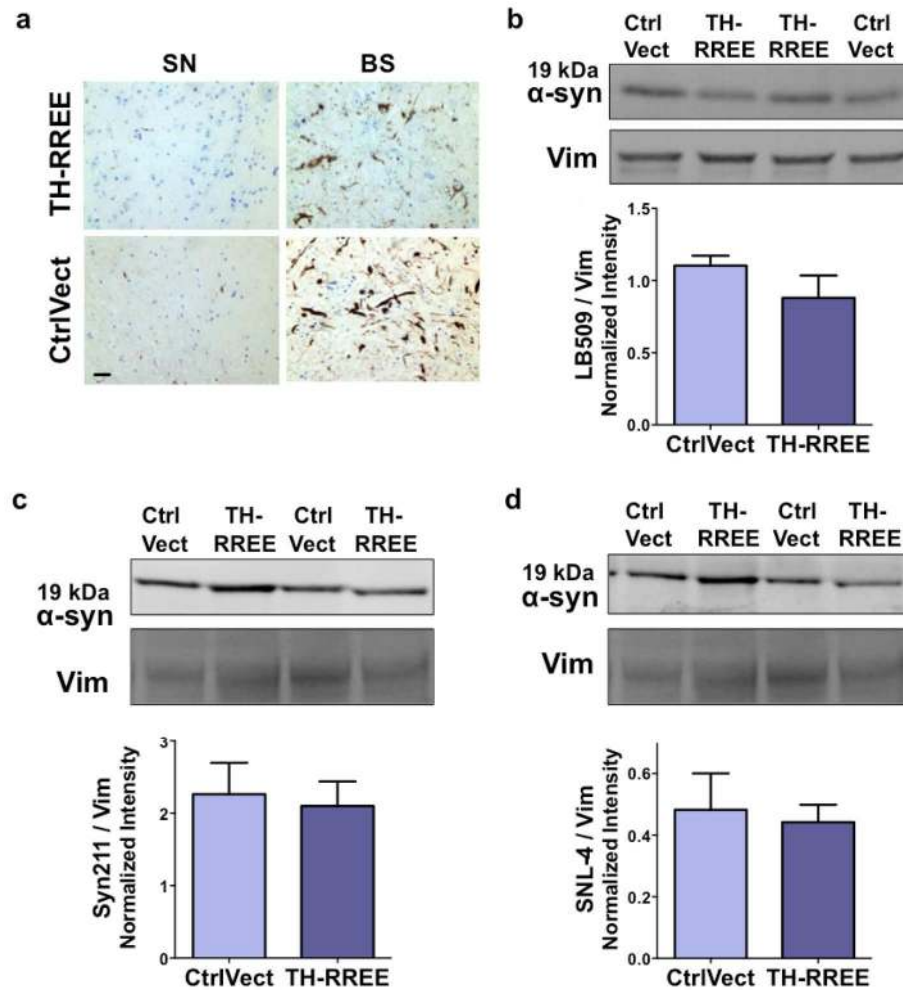
Figure 2.S5: Analysis of inclusion pathology and insoluble α-synuclein in A53T mice. (a) Regardless of lentiviral treatment, α-synuclein inclusion pathology, as detected by Syn505 staining, was abundant in brainstem (BS) and absent in SN of A53T mice at 5 mpi. Scale bar, 20 μm. **(b-d)** At 5 mpi, SN from A53T mice was sequentially extracted with buffers containing 1% Triton followed by 2% SDS. Triton-insoluble/SDS-soluble fractions from A53T TH-RREE and A53T CtrlVect contained similar levels of monomeric α-synuclein as assessed by LB509 **(b)**, Syn211 **(c)**, and SNL-4 **(d)** antibodies. Vimentin (Vim) was used as a loading control, and appears for Syn211 and SNL-4 as the same blot since the same membrane is shown. The data are presented as mean ± s.e.m. (LB509, $n = 3$; Syn211 and SNL-4 $n = 6$ except $n = 4$ for CtrlVect; two-tailed unpaired Student's t test).
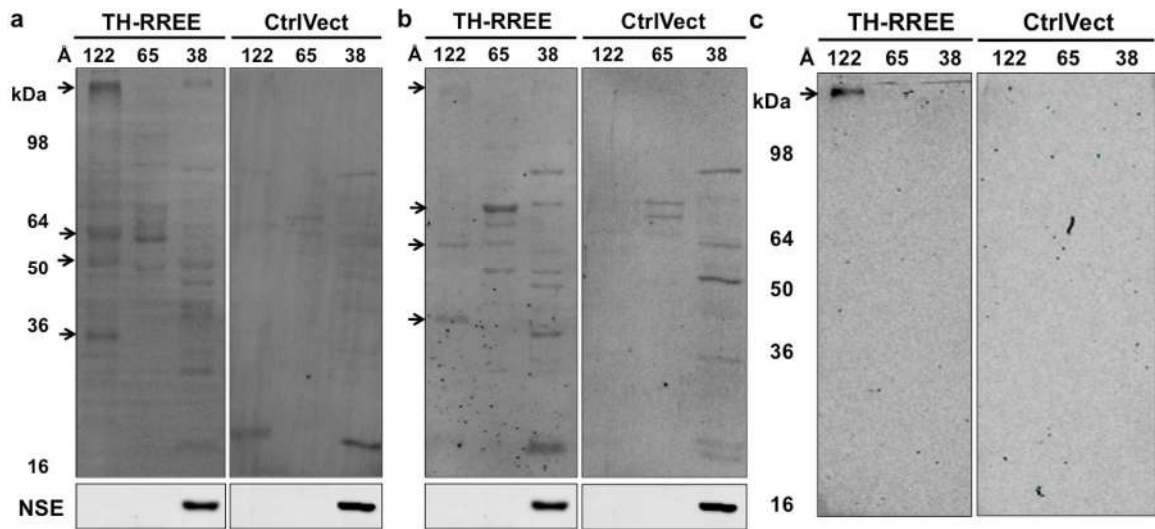
Figure 2.S6: Further characterization of α-synuclein and dopamine derived from A53T mice. (a-b) Triton-soluble SN from A53T TH-RREE and A53T CtrlVect mice at 5 mpi was fractionated by size exclusion chromatography, and the resulting fractions were analyzed by western blot using α-synuclein antibodies Syn211 (C-terminal) **(a)** and SNL-4 (N-terminal) **(b)**. Oligomer bands that uniquely appear in 122 Å fractions from TH-RREE mice are indicated by arrows. Neural specific enolase (NSE) was used as a loading control, and appears for Syn211 and SNL-4 as the same blot since the same membrane is shown. ($n = 4$). **(c)** The SDS-PAGE gels were scanned for near-infrared fluorescence, which revealed oxidized dopamine (arrow) only in 72-122 Å fractions from A53T TH-RREE mice. ($n = 4$).

CHAPTER 3

DYNAMIC STRUCTURAL FLEXIBILITY OF α-SYNUCLEIN

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3.1 Abstract

α-Synuclein is a conserved, abundantly expressed protein that is partially localized in pre-synaptic terminals in the central nervous system. The precise biological function(s) and structure of α-synuclein are under investigation. Recently, the native conformation and the presence of naturally occurring multimeric assemblies have come under debate. These are important deliberations because α-synuclein assembles into highly organized amyloid-like fibrils and non-amyloid amorphous aggregates that constitute the neuronal inclusions in Parkinson's disease and related disorders. Therefore understanding the nature of the native and pathological conformations is pivotal from the standpoint of therapeutic interventions that could maintain α-synuclein in its physiological state. In this review, we will discuss the existing evidence that define the physiological states of α-synuclein and highlight how the inherent structural flexibility of this protein may be important in health and disease.

3.2 Introduction

α-Synuclein is a soluble protein that is highly conserved in vertebrates and abundantly expressed in nervous tissue (Jakes et al., 1994). It was first discovered in 1988 in association with purified synaptic vesicles from the *Torpedo* electric ray (Maroteaux et al., 1988). Soon afterward α-synuclein was found to be widely distributed across the mammalian brain and localized to presynaptic nerve terminals, suggesting functions related to neurotransmission (Iwai et al., 1995). Independent of these reports, α-synuclein was identified as the precursor to a hydrophobic peptide found in Alzheimer's disease senile plaques, termed the non-Aβ component of Alzheimer's

disease amyloid (NAC) (Uéda et al., 1993). The α-synuclein gene was also dynamically regulated during song learning in zebra finch, supporting a role in synaptic plasticity (George et al., 1995).

The discovery of a mutation in the α-synuclein gene that was associated with autosomal dominant inheritance of Parkinson's disease (PD) provided the impetus for a major shift in α-synuclein research (Polymeropoulos et al., 1997). PD is a neuro-degenerative disorder primarily characterized by the loss of dopamine-producing neurons in the substantia nigra pars compacta resulting in motor impairment. Since the original publication of the A53T mutation, several mutations, as well as multiplications of the α-synuclein gene have been linked to PD (Chartier-Harlin et al., 2004; Krüger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Proukakis et al., 2013; Singleton et al., 2003; Zarranz et al., 2004; Ferese et al. 2015) Furthermore, several antibodies against α-synuclein robustly detect the well-known pathoanatomical features of PD, Lewy bodies and Lewy neurites, in postmortem brain tissue from patients with sporadic PD as well as other related neurodegenerative disorders (Baba et al., 1998; Spillantini et al., 1997; Takeda et al., 1998). The finding that wildtype α-synuclein was detected in Lewy bodies and Lewy neurites prompted the publication of numerous studies that investigated the biochemistry and biology of α-synuclein. Despite the rather impressive body of work several fundamental questions remain: What is the physiological function of α-synuclein? What is the structure of native α-synuclein? What factors contribute to the induction of aggregation-competent conformational states of α-synuclein? In this review, we will briefly review the evidence for the different biological functions and discuss ongoing efforts to precisely define physiological structures of α-synuclein.

3.3 The physiological function(s) of α-synuclein

The initial studies indicated that α-synuclein is not required for neuronal development or synapse formation, but instead may modulate synaptic activity. In rodents, α-synuclein is detected close to the time of birth and continues to increase until one month of age, when it reaches a steady-state level that is maintained throughout adulthood (Shibayama-Imazu et al., 1993). Similarly, in cultured rat neurons the development of synapses precedes α-synuclein expression and translocation to axonal terminals (Murphy et al., 2000; Withers et al., 1997). The hypothesis that α-synuclein regulates synaptic activity was directly tested in mice lacking α-synuclein. α-Synuclein null mice develop normal brain architecture and synaptic contacts, and do not exhibit gross behavioral phenotypes (Abeliovich et al., 2000). However, subtle abnormalities in activity-dependent neurotransmitter release have been observed. Upon repeated stimulation, dopaminergic synapses from α-synuclein null mice sustain highly elevated dopamine release (Abeliovich et al., 2000; Yavich et al., 2004). Functional redundancy among α-synuclein and the other synuclein family members, β- and γ-synuclein, may account for the mild phenotypes observed in the single knockout. In α/β-synuclein double knockout mice, synaptic plasticity appears unaltered relative to α-synuclein single knockouts, although dopamine levels in the striatum are reduced (Chandra et al., 2004). The importance of synucleins is particularly highlighted by α/β/γ-synuclein triple knockouts, which have decreased life span and late-onset synaptic dysfunction compared with wildtype mice (Burré et al., 2010; Greten-Harrison et al., 2010). Triple knockouts in another study had motor deficits and decreased striatal dopamine, along with abnormal dopamine neurotransmission (Anwar et al., 2011). Collectively, these reports

emphasize the important role of the synucleins in long-term synaptic maintenance and plasticity.

3.3.1 Synaptic vesicle trafficking

Examination of the role of α-synuclein in the synaptic vesicle cycle has yielded conflicting results. Depletion of α-synuclein from rodent hippocampal neurons both *in vivo* and *in vitro* induces a significant loss of undocked synaptic vesicles, suggesting that α-synuclein acts to replenish or maintain the resting and/or reserve vesicle pools (Cabin et al., 2002; Murphy et al., 2000). In contrast, another study found that increasing α-synuclein in rodent hippocampal neurons reduces the recycling pool of vesicles (Nemani et al., 2010). The effect of α-synuclein on vesicles docked at the plasma membrane prior to exocytosis is similarly unclear. Knockout or knockdown of α-synuclein in rodent hippocampal neurons results in either a decrease or no change in the number of docked vesicles (Cabin et al., 2002; Murphy et al., 2000). Conversely α-synuclein expression in PC12 cells causes an accumulation of vesicles at the plasma membrane and impairment of exocytosis (Larsen et al., 2006). However, in mice modestly overexpressing α-synuclein (levels are not associated with neurotoxicity), hippocampal synapses display a redistribution of vesicles away from the active zone. The density of vesicles in synaptic boutons is also reduced, consistent with α-synuclein-mediated inhibition of vesicle clustering. This is supported by α-synuclein-induced defects in vesicle re-clustering following endocytosis in rat hippocampal neurons (Nemani et al., 2010). Still, opposing results have been obtained from yeast, in which α-synuclein expression results in massive accumulations of vesicles that co-localize with Rab GTPases (Gitler et al., 2008;

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Chen et al., 2008b). Likewise, α-synuclein has been shown to restrict vesicle diffusion away from synapses in mouse hippocampal neurons (Wang et al., 2014). Several lines of evidence, therefore, support the participation of α-synuclein in synaptic vesicle trafficking, though the specific steps for which it may be most important, i.e. vesicle docking, recycling and/or re-clustering, remain unclear.

3.3.2 Chaperone-like activity and neurotransmitter release

α-Synuclein and the other synuclein family members may act as molecular chaperones, facilitating neurotransmitter release. Cysteine-string protein α (CSPα) is a chaperone that is essential for synaptic health; its deletion in mice leads to a decrease in SNARE protein complexes, nerve terminal degeneration, motor impairment and death. When expressed in CSPα-deficient mice, α-synuclein is able to rescue this degenerative phenotype and restore levels of SNARE complexes in synaptic terminals. Moreover, mice lacking both α-synuclein and CSPα exhibit an exacerbated phenotypic decline (Chandra et al., 2005). These findings suggest that α-synuclein is able to complement the activity of CSPα in promoting synapse integrity. Direct evidence for the interaction of α-synuclein with SNARE complexes was documented by co-immunoprecipitation of α-synuclein with SNARE proteins and specific binding to the vesicle-associated SNARE protein synaptobrevin-2. In mammalian cells and purified *in vitro* systems, α-synuclein dose-dependently facilitates SNARE complex assembly (Burré et al., 2010). Additional support for chaperone-like activity includes sequence homology between α-synuclein and 14-3-3 protein chaperones as well as the association of α-synuclein with 14-3-3 and its binding partners in rat brain (Ostrerova et al., 1999). α-, β-, and γ-synucleins are

also able to prevent the aggregation of denatured proteins *in vitro* (Souza et al., 2000a), further supporting a conserved chaperone-like function of synucleins and the existence of several protein-protein interactions that facilitate synaptic function.

3.3.3 Putative role in neurotransmitter synthesis and reuptake

Published evidence indicates that α-synuclein-mediated protein-protein interactions may modulate dopamine synthesis and recycling. α-Synuclein may inhibit the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. α-Synuclein and TH co-immunoprecipitate from rat striatal tissue and MN9D dopaminergic cells and α-synuclein was shown to inhibit TH activity in MN9D and PC12 cells, potentially through PP2A phosphatase-mediated reduction of serine 40 phosphorylation of TH (Peng et al., 2005; Perez et al., 2002). α-Synuclein may also interact with and inhibit the activity of aromatic amino acid decarboxylase, which catalyzes the conversion of L-DOPA to dopamine (Tehranian et al., 2006). Thus, α-synuclein may serve as a negative regulator of dopamine synthesis, though further validation of these findings is necessary. Several reports have also implicated α-synuclein in the regulation of the dopamine transporter (DAT), though the evidence is conflicting with regards to the functional consequences. Direct binding of α-synuclein to DAT has been demonstrated in multiple studies. However, α-synuclein does not appear to alter DAT function, but rather in various cellular contexts can promote or inhibit DAT trafficking to the plasma membrane (Oaks and Sidhu, 2011). Elucidating the relationship between α-synuclein and DAT requires further investigation.

3.4 α-Synuclein structural flexibility

3.4.1 Primary sequence

The primary sequence of α-synuclein consists of 140 amino acids with a predicted

molecular mass of 14,460.16 Da and an isoelectric point of 4.67 (Figure 3.1). The

sequence of α-synuclein is composed of three functionally defined domains. The

N-terminal region (amino acids 1-60) is characterized by the presence of unique and

highly conserved sequence of imperfect tandem repeats with a central consensus motif of

K(A)-**T**(A,V)-**K**(V)-**E**(Q,T)-**G**(Q)-**V**(A). These motifs spanning residues 10-86 are

projected to form two amphipathic α-helices and are characteristic of several proteins

such as apolipoproteins that bind reversibly to membranes (George et al., 1995;

Maroteaux et al., 1988). Indeed the structure of membrane bound α-synuclein contains

two α-helices (amino acids 3-37 and 45-92) in a roughly antiparallel arrangement with a

short linking region (Ulmer et al., 2005). These helices are stabilized by interaction with

a variety of phospholipid bilayers, though α-synuclein interacts preferentially with

membranes of high curvature and an abundance of acidic phospholipids, properties

consistent with those of synaptic vesicles (Davidson et al., 1998; Zhu et al., 2003a).
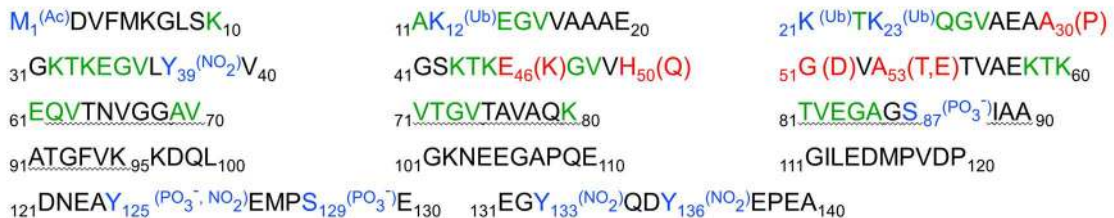
Figure 3.1: Primary sequence of human α-synuclein. Green color indicates the imperfect tandem repeats. Known mutations are indicated in red. The hydrophobic NAC domain is underlined. The major sites of posttranslational modifications identified in vivo are highlighted in blue (Ac, acetylation; Ub, ubiquitination; NO_2, nitration; and PO_3^- phosphorylation).

Upon interaction with membranes of low curvature α-synuclein adopts a distinct secondary structure characterized by a single extended helix that includes both previously described helical domains and the linker region (amino acids 38-44) (Ferreon et al., 2009; Georgieva et al., 2010; Trexler and Rhoades, 2009). All known mutations associated with familial PD (A30P, E46K, H50Q, G51D, A53E, and A53T) are found in the N-terminal domain (Krüger et al., 2008; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004). These mutations, with the exception of G51D, A53E, and A30P, increase the propensity of α-synuclein to form insoluble aggregates and produce morphologically distinct aggregate species (Ghosh et al., 2014; Giasson et al., 1999; Greenbaum et al., 2005; Lesage et al., 2013; Mahul-Mellier et al., 2015; Narhi et al., 1999). Though the precise mechanism by which these mutations promote aggregation has not been conclusively shown, evidence implicate an accelerated formation of oligomers (Conway et al., 2000b) likely due to the destabilization of the native N-terminal conformation (Bertoncini et al., 2005a; Burré et al., 2015; Coskuner and Wise-Scira, 2013; Dettmer et al., 2015).

Amino acids 61-95 compose the hydrophobic NAC domain (Uéda et al., 1993). This region contains a sequence of amino acids (71-82) necessary and sufficient for α-synuclein self-assembly into amyloid fibrils (Giasson et al., 2001). Recently the crystal structures of residues 68-78 (termed NACore), and residues 47-56 (PreNAC) were resolved by the use of micro-electron diffraction, revealing that strands in this region stack in-register into β-sheets that are typical of amyloid assemblies (Rodriguez et al., 2015).

The C-terminal domain (96-140) is rich in negatively charged amino acids (contains 10 glutamate and 5 aspartate residues) and was originally proposed to be essential for maintaining the solubility of the protein. The presence of 5 proline residues, which are known to induce turns and disrupt secondary protein structure, suggested that this region is devoid of secondary structure (George et al., 1995; Ulmer et al., 2005). However, the C-terminus was shown to form transient, long-range interactions with the N-terminus resulting in the formation of multiple compact monomeric structures (Bertoncini et al., 2005a; Dedmon et al., 2005). These compacted structures of α-synuclein are temperature sensitive and are resistant to aggregation. The data also indicated that at elevated temperatures the C-terminus assumes an extended conformation that liberates N-terminal associations and enables aggregation (Bertoncini et al., 2005b; Dedmon et al., 2005). Moreover, C-terminally truncated forms of α-synuclein aggregate faster than full length protein (Hoyer et al., 2004; Li et al., 2005b). Truncated α-synuclein has been detected in the brains of both control (non-disease) and PD patients. Cleavage of full-length protein at residues D115, D119, N122, D125 and Y133 was documented in α-synuclein extracted from LBs (Anderson et al., 2006).

The C-terminus appears to be important for the interaction of α-synuclein with other proteins and for the interaction with small molecules (Burre et al., 2012; Burré et al., 2010; Conway et al., 2001; Mazzulli et al., 2006; Souza et al., 2000b; Woods et al., 2007). Additionally, it contains the major sites of metal binding and post-translational modifications. Binding of iron, copper, and other metals has been shown to influence α-synuclein function and aggregation (Uversky et al., 2001a). Addition of Fe(III), but not Fe(II) to preformed oligomers of α- synuclein accelerates aggregation, raising

the question of metal binding at different points during the aggregation process (Kostka et al., 2008). Cu(II) is unique among metals at accelerating aggregation of α-synuclein at physiologically relevant concentrations. The sole histidine residue H50 in α-synuclein was found to be critical for Cu(II) binding (Rasia et al., 2005) whereas other divalent metal ions, including Mn(II), Co(II), Ni(II) and Fe(II), preferentially bind to the C-terminus of α-synuclein at residues D121, N122, and E123 (Binolfi et al., 2006).

3.4.2 Post-translational modifications

α-Synuclein undergoes a number of post-translational modifications, including N-terminal acetylation, serine and tyrosine phosphorylation, lysine ubiquitination and tyrosine nitration (Oueslati et al., 2010; Barrett & Greenamayer 2015). α-Synuclein purified under mild conditions is acetylated in the N-terminus. The N-terminal acetylation may account for the formation of an oligomeric form of the protein with partial α-helical structure (Trexler and Rhoades, 2012). However, semisynthetic production of N-terminally acetylated α-synuclein demonstrated that modified and unmodified versions of the protein share similar secondary structure, aggregation propensities, and membrane binding (Fauvet et al., 2012b). NMR studies indicated that the first 12 residues undergo a chemical shift due to N-terminal acetylation. This modification also appears to stabilize the helicity of the N-terminus within the context of the full-length protein, and increases the affinity of α-synuclein for lipids (Dikiy and Eliezer, 2014).

Mass spectrometry-based methodologies revealed that α-synuclein extracted from human Lewy bodies was phosphorylated at S129 (Fujiwara et al., 2002). An antibody

raised against phosphorylated S129 was then used to show that α-synuclein was phosphorylated at this site only in subjects with disease and that S129 phosphorylated α-synuclein was present only in the Triton-X- and Sarkosyl-insoluble, urea soluble fraction. These data indicated that some form(s) of aggregated α-synuclein and not the soluble protein is targeted for phosphorylation at S129. Indeed *in vitro* data showed that purified fibrils of α-synuclein are substrates for casein kinase 1 or 2 (Waxman and Giasson, 2008). Other data indicated that polo-like kinase (PLK) 2-mediated phosphorylation of S129 increased autophagy-mediated degradation of α-synuclein, suggesting that phosphorylation may be a neuroprotective mechanism to accelerate clearance of aggregated protein (Oueslati et al., 2013). In addition to the monomeric α-synuclein, S129 phosphorylated bands with apparent molecular weight of 22 kDa and 29 kDa were observed in the detergent insoluble extract (Hasegawa et al., 2002). These bands were also immunoreactive with anti-ubiquitin antibodies suggesting that S129 phosphorylated α-synuclein is also targeted for mono- and di-ubiquitination. It has long been established that the core of Lewy bodies stains positive for both α-synuclein and ubiquitin whereas the surrounding halo is immunoreactive for α-synuclein (Hasegawa et al., 2002). Of the 15 lysine residues in α-synuclein, the major sites of LB-derived α-synuclein undergoing ubiquitination were residues K12, K21, and K23 (Anderson et al., 2006; Hasegawa et al., 2002; Sampathu et al., 2003).

A number of spectroscopic methodologies (CD and NMR) were employed to explore the effect of S129 phosphorylation on the structure of α-synuclein. CD data revealed that phosphorylation of S129 did not affect secondary structure, such that both non-phosphorylated and phosphorylated S129 exhibited random coil structure

(Paleologou et al., 2008). NMR data revealed a number of chemical shifts that occur due to phosphorylation.   While the residues surrounding S129 exhibited the greatest perturbation, residues 1-90 also exhibited detectable chemical shifts (Paleologou et al., 2008) This likely reflects the previously documented long-range interactions of the C- and N-termini. The potential effects of phosphorylation of S129 on the structure of the protein were not faithfully reproduced by mutation of S129 to either E or D, two common phosphomimics used to study the structural consequences of phosphorylation.   For example, phosphorylation at S129 increased the hydrodynamic radius of the protein, whereas S129 E/D mutants did not (Paleologou et al., 2008).

Subsequent studies found additional sites of phosphorylation. Elevated levels of phosphorylated α-synuclein at residue S87 were detected in human brains with Alzheimer's disease, Lewy Body disorders, and multiple system atrophy (Paleologou et al., 2010).   S87 phosphorylation alters the biophysical properties of α-synuclein, including inhibition of fibril formation and reduction in membrane binding (Paleologou et al., 2010).   Additionally, phosphorylated α-synuclein at residue Y125 was detected in Drosophila expressing human wildtype α-synuclein as well as in human brains, though levels were decreased in disease compared with aged-matched healthy controls (Chen et al., 2009).

The proximity of the α-synuclein phosphorylation sites to the metal binding sites raised the question of how phosphorylation may affect metal ion interactions. This was investigated by the use of C-terminal peptides containing residues 119-132 that were either unmodified, phosphorylated at Y125 or at S129 (Liu and Franz, 2005).   By exploiting the luminescence properties of Tb^{3+}, it was found that phosphorylated

Y125 showed enhanced Tb^{3+} binding relative to wildtype or phosphorylated S129. Additionally, phosphorylated Y125 preferentially bound to trivalent rather than divalent metal ions. To investigate this further, longer C-terminal fragments comprised of residues 107-140 that were either unmodified of monophosphorylated at Y125 or S129 were tested for their affinity to various metal ions. By using a fluorescence quenching assay, the dissociation constants of the metal ion complexes and the α-synuclein peptides were determined. These data indicate that either phosphorylation at Y125 or S129 increases the binding affinity for Cu (II) and Fe(II), but not Fe(III). Furthermore, phosphorylated Y125 has a greater affinity for Pb(II) than wildtype, but phosphorylated S129 has an even greater affinity than phosphorylated Y125. Additionally, tandem MS indicated that phosphorylation causes the metal ion binding sites to shift towards the C-terminal end of α-synuclein (Lu et al., 2011).

α-Synuclein within Lewy bodies is nitrated on all four tyrosine residues (Giasson et al., 2000). Chemical nitration of α-synuclein results in the formation of both tyrosine nitrated monomers and nitrated dimers (Souza et al. 2000b). Immunoelectron microscopy confirmed that nitrated monomers and dimers are incorporated into amyloid fibrils. Purified nitrated α-synuclein monomer by itself was unable to form fibrils, whereas the nitrated dimer accelerated aggregation of unmodified α-synuclein (Hodara et al., 2004). Additionally, nitration at residue Y39 in the N-terminus decreased binding to synthetic vesicles and prevented the protein from adopting α-helical conformation (Hodara et al., 2004). These observations were recently confirmed and elegantly expanded by the generation of site-specifically nitrated α-synuclein using protein semisynthetic chemistries (Burai et al. 2015). Using the synthetic nitrated α-

synuclein the data showed that nitration did not interfere with phosphorylation of S129 by PLK3 and reaffirmed that intermolecular interactions between the N- and C-terminal regions of α-synuclein are critical in directing nitration-induced oligomerization of α-synuclein (Burai et al. 2015).

3.4.3 Native conformation(s) of α-synuclein

Figure 3.2 depicts the rapid growth in the number of publications identified in PubMed using the term synuclein and highlights key studies that explored the native structure and conformation of the protein. Early biochemical studies of α-synuclein isolated from bacterial expression systems or α-synuclein expressed in rodent tissues indicated that it is monomeric with limited secondary structure. Electrophoretic
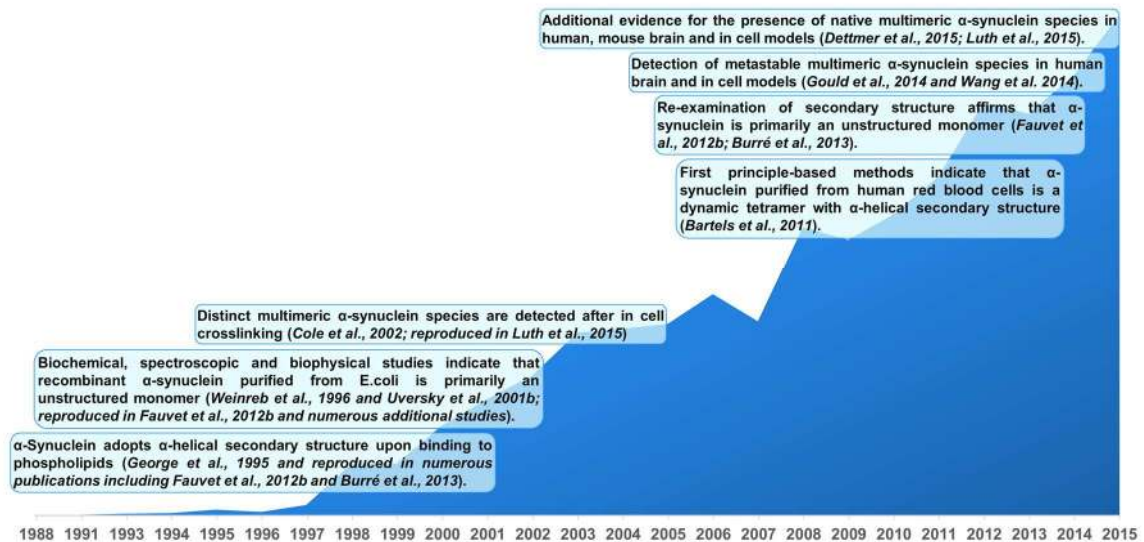
Figure 3.2: The graph depicts the number of publications retrieved from PubMed using the search term "alpha synuclein" from a single publication in 1998 to 862 in 2015. Significant milestones that examined the native structure and conformations of α-synuclein are displayed.

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separation of α-synuclein purified without heating on 6, 10, or 14% acrylamide gels estimated an apparent molecular weight of 20±3 kDa. However, the values of sedimentation coefficient ($S_{20,w}$ = 1.7S), stokes radius (34 Å), analysis on native gels and derivation of the frictional coefficient (f/f_o=2.09) indicated an apparent molecular weight in the range 57-58 kDa (Weinreb et al., 1996). To reconcile this apparently anomalous behavior it was proposed that monomeric α-synuclein achieves minimal structure in simple solutions and this rather extended unstructured conformation resembles a globular protein with a larger apparent molecular weight. This assumption was further corroborated by examination of purified monomeric α-synuclein by CD, FTIR and small angle X-ray scattering, which failed to identify significant secondary structural features. Furthermore, minimal shifts in the spectroscopic features of α-synuclein were observed when the protein was placed in solutions that would increase hydrophobicity and neutralize negative charges indicating that the protein is natively unstructured, joining a growing group of proteins sharing similar biochemical and biophysical characteristics (Uversky et al., 2001b). NMR and CD data, however, indicated that α-synuclein assumes increasingly folded secondary structure when exposed to conditions that promote aggregation (low pH and high temperature) or upon interaction with phospholipids. Collectively these data indicated that native α-synuclein is primarily an unstructured monomer, which can assume different compact conformations that resist aggregation, adopts α-helical conformation upon binding to lipids and undergoes conformational changes prior to oligomerization and formation of amyloid fibrils (Uversky et al., 2001b). However, the methodologies employed to quantify the molecular weight of α-synuclein in these elegant studies were not based on first principles and therefore a lingering

uncertainty remains regarding the native size of the protein. Moreover, crosslinking experiments in both intact cells expressing α-synuclein and lipid-free lysates revealed the stabilization of high molecular weight α-synuclein multimers (consistent with dimers, trimers, and larger multimers). These multimers were not reduced by dilution of lysates before crosslinking, nor by reducing the concentration of crosslinker from 1 mM to 8 μM, suggesting that they represented endogenous protein complexes (Cole et al., 2002).

Examination of the α-synuclein native state was reignited in 2011 with the publication of results indicating that α-synuclein exists natively as a tetramer, rather than a monomer. Methodologies that are based on first principles were employed to examine the molecular weight and size of α-synuclein extracted under non-denaturing conditions from human red blood cells. Analytical ultracentrifugation produced a sedimentation equilibrium value of 4.78 S, indicating a molecular weight of 57.8 kDa. Analysis of particle geometry by scanning transmission electron microscopy revealed the presence of roughly spherical molecules with a diameter of approximately 3.0-3.5 nm. Automated sampling of 1000 α-synuclein particles showed a distribution of molecular weights between 10 and 175 kDa with a peak distribution at 55 kDa. These findings constitute the most direct measurements of the native molecular weight of α-synuclein. The tetrameric species were shown to have α-helical conformation and were resistant to aggregation (Bartels et al., 2011).

Complimentary observations were made using recombinant GST-tagged α-synuclein purified from bacterial expression systems under non-denaturing conditions. Single-particle electron microscopy of purified α-synuclein revealed complexes of sizes and internal geometries consistent with trimers and dimers, which were

corroborated by measurements of the hydrodynamic radii and elution on native state PAGE. As observed previously, these species were more resistant to aggregation than denatured monomer. CD also showed that several α-synuclein mutations associated with early onset PD (A30P, E46K, A53T) exist in less ordered conformations than wildtype α-synuclein. These mutants were also more prone to aggregation (Wang et al., 2011). However, using the same α-synuclein construct that contains a 10-residue N-terminal extension, which forms multimers when isolated from E. coli, NMR studies indicated that only a small fraction of α-synuclein assembles into α-helical trimers and tetramers and the majority remains as a disordered monomer (Gurry et al., 2013). These data indicated that several potential conformers of α-synuclein may exist in equilibrium. The observation that α-helical trimers and tetramers constitute only a small fraction of the total α-synuclein may explain other studies in which in-cell NMR was used to probe for the structure of α-synuclein and reported primarily the presence of unstructured monomer. NMR data of α-synuclein in intact cells failed to detect stable or highly populated α-synuclein multimers and confirmed the intrinsically disordered nature of the protein in *E. coli* regardless of its purification method (Binolfi et al., 2012). Collectively these studies generated an apparent controversy and stimulated several additional studies that explored the native size and structure of α-synuclein.

A re-examination of the native state of α-synuclein reasserted that the behavior of α-synuclein from various sources was consistent with a disordered monomer. This behavior was observed with protein extracted and isolated under both denaturing and non-denaturing conditions. CD spectra previously attributed to tetrameric assemblies were not reproduced using isolated monomer, but were replicated with the

addition of small unilamellar vesicles. Natively isolated α-synuclein before or after boiling that disrupts secondary structure migrated as high molecular weight α-synuclein bands in native PAGE, which was attributed to the rather expanded size of the unstructured monomer in solution. These findings reaffirmed that the majority of native α-synuclein is a monomer with minimal secondary structure (Fauvet et al., 2012a). Further support was provided by similar explorations in the mouse brain, which indicated that the predominant native form of α-synuclein is an unstructured monomer. α-Synuclein exhibited random coil structure in solution, readily aggregated over time, and adopted α-helical structure only upon membrane binding (Burré et al., 2013).

α-Synuclein multimers were detected in postmortem non-diseased human brain using mild protein extraction methods, but no further purification. These α-synuclein multimers had Stokes radii ranging from 33.2-37.5 Å, sedimentation coefficients ranging from 1.4S to 3.8S and apparent molecular weights ranging from 53-70 kDa in native gradient gels. The multimers were detected by anti-α-synuclein antibodies that recognize different epitopes and the multimer identity was confirmed by mass spectrometry. Consistent with previous observations, melting point thermostability analysis showed progressive loss of the α-synuclein multimers and heating of the brain extracts above 55°C collapsed the higher molecular weight α-synuclein conformers into the 53 kDa species, which corresponds to the unstructured monomer. These data indicated the presence of α-synuclein conformers, defined as conformationally diverse α-synuclein multimers, in the human brain. Therefore it appears that both monomer and metastable multimers coexist and that interactions with lipids, other proteins, or small molecules may transiently stabilize these species (Gould et al., 2014). This was further

supported by controlled bimolecular fluorescence complementation methodologies in different cell types that found α-synuclein metastable conformers assembled in synapses. It was suggested that the function of these multimeric α-synuclein conformers is to restrict recycling of synaptic vesicles and thus reduce neurotransmitter release (Wang et al. 2014).

Additional support for native multimeric species comes from recent studies in which serial purification of α-synuclein from non-pathological human cortical tissue was performed. Removal of lysate components other than protein followed by sequential removal of proteins though size exclusion, anion chromatography, and thiopropyl sepharose 6b separation, resulted in the isolation of >90% pure α-synuclein. Each step of serial purification resulted in a progressive loss of α-synuclein immunoreactive high molecular weight bands observed after disuccinimidyl glutarate crosslinking and SDS-PAGE separation. Analysis of α-synuclein secondary structure by CD found that the sequentially purified protein had greater α-helical content than the recombinant α-synuclein. However, a high degree of variability in secondary structure was observed between purified samples raising questions about the stability of these helical conformations (Luth et al., 2015). Furthermore, crosslinking experiments conducted in brain tissue from mice expressing wildtype or A53T human α-synuclein in the absence of mouse α-synuclein showed that the A53T mutation reduced the presence of soluble multimeric α-synuclein (Dettmer et al., 2015).

3.5 Concluding remarks and perspectives

Collectively the studies on the native structure indicate a remarkable conformational plasticity and structural flexibility of α-synuclein. The ability of the protein to adopt N-terminal α-helical conformation through its association with lipids has been well documented. The association with lipids has been shown to prevent fibril formation (Martinez et al., 2007; Zhu and Fink, 2003b) and may also stabilize physiological multimeric species that together with the monomer regulate SNARE complex assembly and recycling of synaptic vesicles (Burré et al., 2014; Wang et al. 2014). However, other groups have demonstrated a role for phospholipid membranes in promoting pathological α-synuclein aggregation, potentially by acting as a scaffold for amyloid nucleation. This event may preferentially occur at low lipid to protein ratios, when monomeric α-synuclein is free in solution and can participate in nucleation (Galvagnion et al., 2015; Ysselstein et al., 2015).

In Figure 3.3 we propose a model which incorporates and summarizes the existing knowledge regarding α-synuclein biology and structure. The steady state levels of α-synuclein are carefully regulated by protein synthesis and removal by several pathways such as the ubiquitin-proteasome pathways and autophagy (Webb et al., 2003). Controlling the steady state levels of this protein by regulating synthesis and degradation may be the first critical defense in preventing aggregation. Conformational change to α-helical rich structures, and stabilization of metastable multimers is achieved by specific interactions with vesicular phospholipids and proteins. The sequestration of α-synuclein in association with membrane vesicles and with other proteins may be of critical importance for preventing aggregation. Therefore these dynamic equilibria
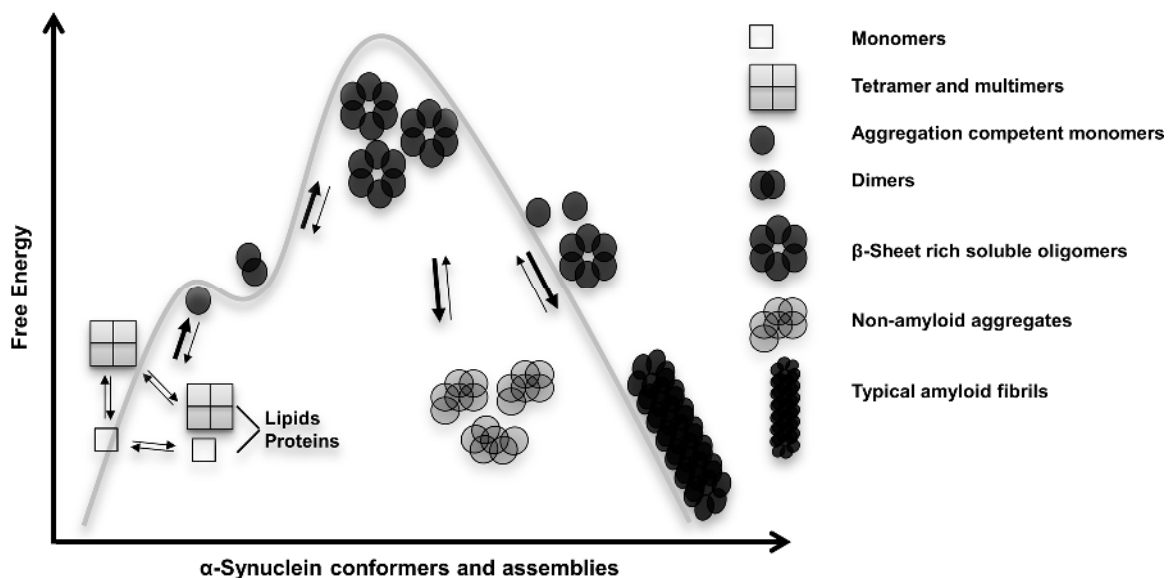
Figure 3.3: Free energy landscape of possible α-synuclein conformers and multimeric assemblies. The conversion of native α-synuclein to aggregation-competent monomers may depend on dissociation from stabilizing interactions with lipids and/or proteins as well as dissociation of the metastable tetrameric species. α-Synuclein aggregation-competent monomers can then assemble into dimers and larger oligomeric conformers. The generation of α-synuclein oligomers can rapidly lead to formation of stable amyloid fibrils, or 'off-pathway' amorphous aggregates, both of which have been observed in postmortem brain tissue from patients with PD and related disorders.

maintain functionality and promote assemblies that are resistant to aggregation. Catastrophic events that may include inappropriate post-translational modifications will disassemble the multimers as well as transform aggregation-incompetent monomers to aggregation-competent species. The first step in the pathway to amyloid fibril formation is the generation of a dimer that is either held together by hydrophobic interactions induced by increased conformational transition to β-sheet structure or upon covalent cross-linking. Following this nucleation event (Wood et al., 1999) the hydrophobic patch of amino acids between residues 71-82 appears to be primarily responsible for allowing additional α-synuclein monomers to assemble to form oligomeric structures.

This transition is the committed rate limiting step for aggregation and must overcome a relatively large thermodynamic requirement that permits the conversion from an unstructured coil to organized β-sheet conformation. Oligomers are soluble in aqueous buffers and can appear spherical or ring-like by atomic force and electron microscopy (Conway et al., 2000b; Lashuel et al., 2002). Soluble, high molecular weight oligomers have been extracted from human brain tissue and their levels appear to be increased in PD brain (Sharon et al., 2003) as well as mouse models of α-synuclein aggregation (Tsika et al., 2010). As oligomers grow, they reach an undefined critical length and are able to assume additional quaternary structure. At this stage, these structures may continue to grow in linear β-sheets, forming polarized protofibrils and eventually fibrils. Fibrils may further arrange into protein inclusions although it remains unclear if other proteins within these inclusions anchor these fibrils. Alternatively, oligomers may remain soluble by interacting with small molecules (Conway et al., 2001) or by incorporating post-translationally modified α-synuclein molecules. These structures remain "off the amyloid fibril pathway" and may constitute what has been described in human postmortem tissue as "dots" or "dust-like" amorphous aggregates (Braak et al., 2001; Duda et al., 2002). At this juncture, it remains unclear which of these assemblies are toxic to neurons. Recent data indicate that several conformationally distinct assemblies (possibly different strains) of α-synuclein generated *in vitro* will induce the aggregation of endogenous α-synuclein resulting in neurodegeneration (Guo et al., 2013; Luk et al., 2012; Peelaerts et al., 2015; Sacino et al., 2014). The appreciation of different α-synuclein conformers and assemblies as well as their roles in disease may guide potential therapeutic approaches. For example, therapeutic strategies can be centered on preserving and stabilizing the

physiological multimeric conformers as well as preventing monomers from aggregating. Alternatively, sequestration and removal of aggregation-competent monomers and oligomers can be considered.

3.6 Acknowledgements

CHAPTER 4

SUMMARY AND DISCUSSION

A pathognomonic hallmark of idiopathic PD is the Lewy body, an intraneuronal inclusion containing aggregated α-synuclein. Lewy pathology develops in circumscribed brain regions, and neurodegeneration occurs in several of these areas including the SN (Forno, 1996; Braak et al., 2003). Coupled with the discovery that mutations and multiplications of the α-synuclein gene cause rare forms of familial PD (Goedert et al., 2013), these defining features suggest that α-synuclein is a major player in PD pathogenesis. The correlation between Lewy body inclusions and neuronal cell death is imperfect, however. Not all neuronal populations that harbor α-synuclein inclusions also exhibit marked cell loss (Forno, 1996). Furthermore the density of inclusions does not correlate well with clinical severity (Ross and Poirier, 2005). Lewy bodies have also been found in the brains of some elderly asymptomatic individuals (Goedert et al., 2013). Mutations in LRRK2, a common cause of familial PD, are not always associated with Lewy pathology, and juvenile-onset parkinsonism typically develops in the absence of α-synuclein inclusions (Goedert et al., 2013). These observations highlight the need to better understand the role of α-synuclein aggregation in PD etiology, and raise the possibility of α-synuclein-independent disease mechanisms.

In sporadic PD, which constitutes the majority of cases, the presence of Lewy bodies in addition to smaller aggregates of α-synuclein has made it difficult to identify which species, if any, are primarily responsible for neuronal degeneration. Lewy bodies and Lewy neurites could present enormous challenges to normal cellular functions, including protein degradation, axonal transport, and maintenance of the cytoskeleton (Norris et al., 2004). It is possible that all neurons burdened with α-synuclein deposits are on a trajectory towards cell death, but due to region- and/or cell type-specific

factors, neuronal dysfunction and loss manifest with varying kinetics. This may explain the imperfect correlation between Lewy pathology and neurodegeneration or clinical symptoms, and suggests that individuals with incidental Lewy body disease may have preclinical PD (Goedert et al., 2013). Alternatively, inclusion bodies may serve to protect neurons by sequestering smaller α-synuclein aggregates such as oligomers or fibrils, for which there is increasing evidence of toxicity (Danzer et al., 2007; Karpinar et al., 2009; Tsika et al., 2010; Volpicelli-Daley et al., 2011; Winner et al., 2011; Luk et al., 2012a). In Chapter 2 of this thesis, I present evidence for a new mouse model of progressive SN neurodegeneration and locomotor deficit driven by α-synuclein toxicity. This model was used to examine the relationship of biochemically and histologically defined α-synuclein aggregates with disease, and I found that dopaminergic neurons degenerated in the absence of Lewy body-like inclusions. Instead, the disease phenotype correlated with increased levels of oligomers, which bore similar conformations to neurotoxic oligomers generated *in vitro*. Thus, α-synuclein aggregation in multiple brain regions may give rise to PD specifically through the formation of oligomers. Moreover, the dependence of both the observed degeneration and oligomer species on dopamine suggests that catecholamines may enhance α-synuclein toxicity and confer heightened vulnerability to SN neurons.

Several genes linked to familial PD also implicate mitochondrial dysfunction in neurodegeneration of the SN and development of parkinsonian signs. Genetic mutations in Parkin, PINK1, and DJ-1 may converge on a common pathway in which the normal maintenance and turnover of mitochondria is disrupted (Hernandez et al., 2016). This may lead to energy depletion, and oxidative stress through increased production of

reactive oxygen species (Bose and Beal, 2016). Dopaminergic neurons in the SN may be particularly susceptible to oxidative damage due to the normal metabolism and auto-oxidation of dopamine (Graham, 1978; Fahn and Cohen, 1992). In support of these mechanisms, complex I inhibitors such as MPTP induce oxidative stress and reproduce PD-like dopaminergic cell death in both humans and animal models (Dauer and Przedborski, 2003). Knockout of Parkin, PINK1, or DJ-1 has been largely insufficient to induce SN neuron loss in mice (Blesa and Przedborski, 2014), although this may reflect the slow progression of autosomal recessive PD (Goedert et al., 2013). Critically, the lack of Lewy body pathology in such cases of PD suggests that this form of the disease may be mechanistically distinct from the typical late-onset disorder. Toxicity associated with α-synuclein aggregation may play a more central role in idiopathic PD, although a potential contribution of α-synuclein to mitochondrial impairment cannot be discounted since α-synuclein knockout mice are resistant to MPTP (Dauer et al., 2002). The mouse model presented in Chapter 2 can be used to further investigate potential interactions between mitochondria, α-synuclein, and dopamine. In addition, rescue of the degenerative phenotype by manipulation of putative disease mechanisms, for example through pharmacological or genetic disruption of toxic interactions or upregulation of neuroprotective pathways, will inform new strategies for PD treatment.

4.1 The synergistic interaction between dopamine and α-synuclein drives SN neurodegeneration

In Chapter 2, my findings indicate that nigrostriatal degeneration is dependent on the convergence of two factors: elevated dopamine and expression of α-synuclein.

In the mouse brain neither of these factors alone was sufficient to induce neurotoxicity, suggesting that it is their specific interaction that is required. Given the postulated role of dopamine in promoting oxidative stress (Graham, 1978; Fahn and Cohen, 1992; Jenner and Olanow, 1996; Stokes et al., 1999), it is somewhat surprising that long-term elevation of dopamine levels in NonTg mice did not result in dopaminergic nerve terminal or cell body loss. Lentiviral-mediated expression of a dysregulated mutant TH achieved a 52% increase in total striatal dopamine content measured at 5 mpi. It is possible that excess dopamine was well-tolerated due to compensatory upregulation of metabolic enzymes and transporters; indeed, there was a significant increase in DAT levels in these mice. Oxidative stress may be prevented if dopamine storage, release, and re-uptake mechanisms remain tightly coupled with dopamine levels, for example through the efficient cycling of excess dopamine into acidified vesicles where its kinetics of oxidation are much slower than in the cytosol. To my knowledge, this is the first *in vivo* report of a sustained increase in dopamine synthesis and the effects on neuronal health. Previous studies have shown that single, high dose injections of dopamine into the striatum of rats can be acutely toxic within the short timeframe of 10 days (Filloux and Townsend, 1993; Hastings et al., 1996; Rabinovic et al., 2000). Other groups have demonstrated neurotoxicity associated with redistribution of endogenous dopamine to the cytosol by manipulation of VMAT2 or DAT in mice (Colebrooke et al., 2006; Caudle et al., 2007; Chen et al., 2008a; Masoud et al., 2015). While dopamine has been implicated in PD pathogenesis, its precise role has remained poorly defined.

Despite the clear link between α-synuclein misfolding/aggregation and neurodegenerative disease in humans, it has been difficult to recapitulate these

features in transgenic animal models. The overexpression of human WT or familial mutant α-synuclein in mice has often resulted in a lack of inclusion pathology and neurodegenerative changes in the SN (Rathke-Hartlieb et al., 2001; Matsuoka et al., 2001; Richfield et al., 2002; Giasson et al., 2002; Lee et al., 2002; Gispert et al., 2003; Gomez-Isla et al., 2003; Yavich et al., 2005; Martin et al., 2006; Emmer et al., 2011). It is well-established that dopaminergic neurons are entirely spared in the A53T transgenic mice used in the present work (Giasson et al., 2002) and I also documented a normal complement of neuronal cell bodies and terminals and a lack of intraneuronal α-synuclein inclusions in the SN in these mice. This may be related to several factors, including the fact that WT mouse α-synuclein allele has a threonine at position 53. *In vitro*, mouse α-synuclein fibrillizes more rapidly than human WT or A53T α-synuclein, and yet aged mice do not spontaneously develop α-synuclein inclusions or a PD-like disorder (Rochet et al., 2000). It is likely that mice have evolved protective mechanisms against α-synuclein aggregation, such as an enhanced ability to maintain α-synuclein in a physiological lipid- or protein-bound state, and that these mechanisms may counteract efforts to initiate disease by transgenic α-synuclein expression. Given that age is the greatest risk factor for PD, another possibility is that the short lifespan of mice (typically 1-2 years) does not allow adequate time for development of PD-like inclusions or neurodegeneration with α-synuclein overexpression.

Strikingly, elevating total striatal dopamine in A53T mice by TH-RREE lentiviral injection resulted in progressive nigrostriatal degeneration and a locomotor deficit, suggesting that dopamine dysregulation is a critical factor in α-synuclein mediated toxicity. Following an early decrease in dopaminergic nerve terminals, A53T

TH-RREE mice exhibited a more severe loss of terminals and overt neuronal cell loss in the SN at five months after injection. Moreover, there was a time-dependent decline in dopamine levels that led to a significant reduction in ambulation. These findings are consistent with other studies in which dopamine and α-synuclein act synergistically to compromise cellular health. In the case of VMAT2-deficient mice, loss of SN neurons was only observed in animals on a normal α-synuclein background, whereas identical reduction of VMAT2 in animals with an α-synuclein gene locus deletion did not result in cell loss (Specht and Schoepfer, 2001; Colebrooke et al., 2006; Caudle et al., 2007). In rodent midbrain cultures, elevating cytosolic dopamine concentration reduced dopaminergic cell survival and this effect was dependent on α-synuclein (Mosharov et al., 2009). Similarly, both dopamine and α-synuclein were required to induce cell death in human fetal dopaminergic cultures and SH-SY5Y cells (Xu et al., 2002).

The progressive nature of the phenotype in A53T TH-RREE mice in which dopaminergic terminals appear to undergo degeneration prior to cell bodies is supported by existing evidence that synapses are an early site of the disease process. Autopsy reports as well as PET imaging studies in living PD patients suggest that, at the time of disease onset, the loss of striatal nerve terminals exceeds that of dopaminergic cell bodies (Cheng et al., 2010). Axonal pathology containing α-synuclein has also been identified in PD and DLB (Galvin et al., 1999). Consistent with the human data, expression of human α-synuclein in the rat SN induced synaptic dysfunction and axonal damage that preceded substantial cell loss (Chung et al., 2009; Lundblad et al, 2012). My results show that at the early timepoint of 2.5 mpi, there is a documented 25% loss of dopaminergic nerve terminals in A53T TH-   RREE mice, without an accompanying

reduction of neuronal cell bodies in the SN. Axonal degeneration progresses such that dopaminergic innervation of the striatum falls to 62% of age-matched controls, and coincides with a 25% loss of neurons in the SN. These data are in line with a disease mechanism that begins at the synapse, causes a 'dying back' of axons and a retrograde progression of neurotoxic events that ultimately lead to neuronal demise. Indeed, the localization of both dopamine and α-synuclein to presynaptic terminals permits a direct interaction that may drive neurodegeneration.

4.2 Oligomers are a potential point of convergence for dopamine and α-synuclein neurotoxicity

The aggregation of α-synuclein can produce several intermediate species and both fibrillar and non-fibrillar products, and it has been difficult to identify which species may be relevant to disease. α-Synuclein oligomers are thought to be critical players in PD and other synucleinopathies (Ross and Poirier, 2005). Oligomeric species have been identified and linked with toxicity in cell culture (Gosavi et al., 2002; Outeiro et al., 2008) and animal models (Tsika et al., 2010; Winner et al., 2011), and appear to be increased in PD brain (Sharon et al., 2003). Dopamine has been shown to promote α-synuclein oligomerization both in purified protein systems and cell cultures, through the non-covalent interaction of oxidized dopamine with the C-terminal 125-YEMPS-129 motif in α-synuclein (Conway et al., 2001; Norris et al., 2005; Mazzulli et al., 2006; 2007). In Chapter 2, I report the novel finding that dopamine increases and modifies α-synuclein oligomers in the mouse brain, suggesting that dopamine may act as an enhancer of oligomer toxicity. Presumably, these changes are mediated by cytosolic

dopamine, which could encounter and directly interact with α-synuclein, though the possibility of indirect mechanisms involving vesicular or extracellular dopamine cannot be excluded. The relevant source of dopamine may be identified by manipulation of dopamine compartmentalization, for example by co-overexpression of VMAT2, which would be expected to mitigate α-synuclein oligomerization and neurodegeneration.

Consistent with dopamine-mediated stabilization of α-synuclein oligomers *in vitro* (Conway et al., 2001; Norris et al., 2005), elevating total striatal dopamine in A53T mice resulted in greater levels of oligomeric species. It appears that these species were primarily generated from monomeric α-synuclein rather than larger aggregates, as monomer levels were significantly decreased and insoluble α-synuclein remained unchanged. While it has been reported that dopamine is able to disaggregate α-synuclein fibrils (Li et al., 2004), this remains to be established and I did not find evidence of disaggregation in our mouse model. Rather, it is more likely that dopamine acts to shift the dynamic equilibrium between monomers and oligomers in favor of the oligomeric state. This could be achieved, for example, through direct interaction with α-synuclein oligomers, effectively removing them from the reaction, or by interaction with monomers such that they become aggregation-competent. At present, the exact α-synuclein species with which dopamine interacts is unknown, although the presence of oxidized dopamine only in high molecular weight protein complexes extracted from A53T TH-RREE mice suggests that oligomers may be the likely target.

The oligomer species that result from the interaction of dopamine and α-synuclein *in vivo* assume both similar and distinct conformations from those previously observed. In the SN of A53T control mice, soluble   oligomers with Stokes radii of up to 65

Å and apparent molecular weights ranging from 36 to 80 kD were identified, entirely consistent with previous biochemical characterization (Tsika et al., 2010). These low Å oligomers were also found in A53T TH-RREE mice and appeared more abundant than in control mice. Interestingly, larger species between 72-122 Å were present only in A53T TH-RREE mice, and were detected using multiple α-synuclein antibodies by both western blotting and immunoelectron microscopy. The increase in oligomer size is in line with the previous observation by atomic force microscopy that larger diameter particles are produced *in vitro* when α-synuclein is incubated with dopamine than without (Follmer et al., 2007). The significance of these findings is two-fold: for the first time it is demonstrated that dopamine promotes α-synuclein oligomerization *in vivo*, and moreover that the resultant species possess unique conformations. The effects of dopamine on α-synuclein aggregation may produce neurotoxic species that ultimately drive neuronal cell death.

The toxicity of dopamine-induced α-synuclein oligomers was tested using recombinant protein that had been incubated with dopamine *in vitro*. Soluble, high molecular weight oligomers resembling mouse-derived species were generated, and biochemical characterization recapitulated earlier findings (Norris et al., 2005; Cappai et al., 2005; Pham et al., 2009). When applied to primary hippocampal cultures, the recombinant oligomers dose-dependently reduced neuronal viability, whereas neither oxidized dopamine nor monomeric α-synuclein had the same effect. It is important to note that several aspects of this experimental paradigm diverge from the mouse model, including the non-dopaminergic cell type, and the extracellular application of oligomers. While hippocampal neurons do not mimic   exactly the physiology and metabolism

of dopaminergic cells, they nonetheless offer a neuronal model in which to test the sufficiency of α-synuclein oligomers to incur toxicity. The observation that dopamine-induced oligomers were indeed toxic suggests that mechanisms of cell death downstream of the dopamine-α-synuclein interaction are not dependent on a dopaminergic cellular phenotype. Regarding the exogenous source of α-synuclein oligomers, there is growing evidence that α-synuclein aggregates are secreted into the extracellular space and taken up by neighboring cells where they propagate disease pathology (Lee, 2008; Goedert et al., 2010).   There is also some evidence that dopamine promotes the secretion of α-synuclein oligomers (Lee et al., 2011). Exogenous α-synuclein was detected inside neurons following oligomer treatment, suggesting that dopamine-induced species are able to translocate across cellular membranes and gain access to nearby cells. A model for the intracellular formation of these species and possible mechanisms of cell-to-cell spread is outlined in Figure 4.1.

An alternative approach to test dopamine-induced oligomer toxicity is to disrupt the interaction between α-synuclein and dopamine, and examine if neuronal loss is mitigated. This could be achieved by expressing a mutant α-synuclein lacking the C-terminal 125-YEMPS-129 motif that is required for dopamine-mediated stabilization of oligomers (Norris et al., 2005; Mazzulli et al., 2007). The small model organism *C. elegans* could be utilized for its powerful attributes: a genetically tractable system in which the entire genome has been sequenced and all neuronal identities have been mapped, a short generation time and lifespan, transparency which allows for the imaging of selected cells in living animals, and a repertoire of behaviors that have been associated with particular neurons or neurotransmitter   systems (Corsi et al., 2015). *C. elegans*
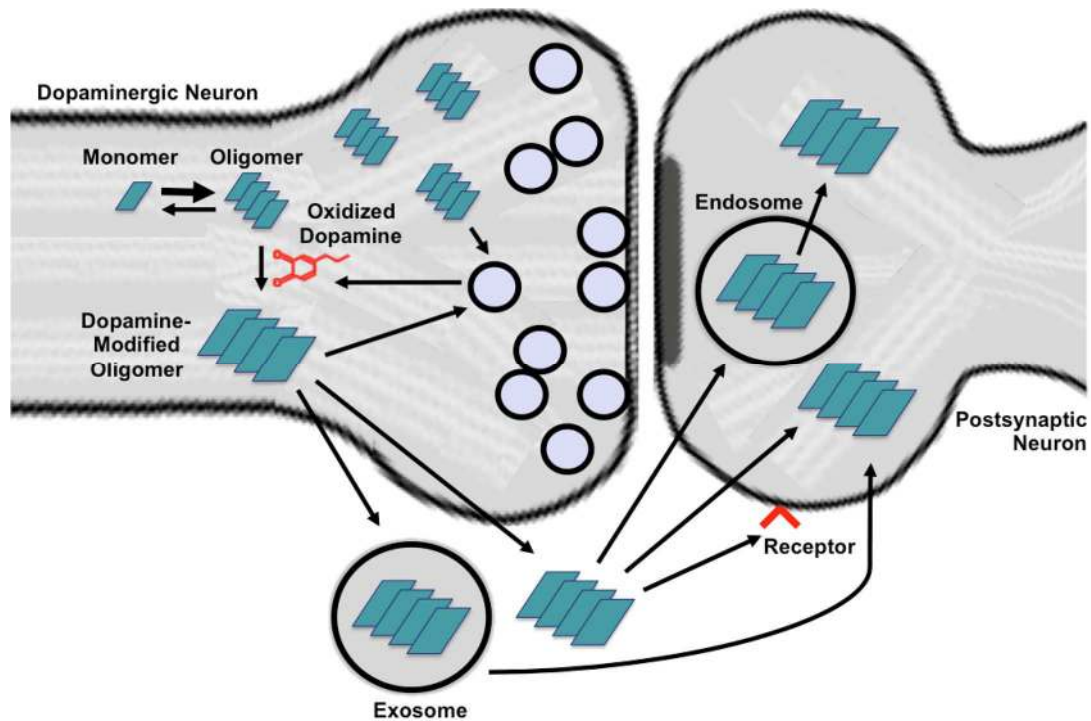
Figure 4.1: Model for cell-to-cell transfer of dopamine-induced oligomers. In disease, α-synuclein monomers may be in dynamic equilibrium with aggregated species such as oligomers. Oxidized dopamine in the cytosol may directly interact with oligomers resulting in modified conformations with enhanced toxicity. Dopamine-mediated stabilization of these species may shift the equilibrium towards the formation of additional oligomers, causing an increase in the total steady-state levels of oligomeric α-synuclein. Dopamine-modified oligomers may propagate by disruption of synaptic vesicle membranes, allowing dopamine to leak into the cytosol and interact further with oligomers. These species may also disrupt the plasma membrane gaining access to the extracellular space, and then directly enter neighboring neurons or become endocytosed, potentially via receptor-mediated endocytosis. Oligomers may further escape endosomes by aberrant membrane interactions. Alternatively, dopamine-modified oligomers may be actively released by dopaminergic neurons by exocytosis, and then taken up by other neurons. Due to the non-covalent nature of the interaction between α-synuclein and dopamine, oligomers may eventually be able to participate in the formation of Lewy bodies. The cell-to-cell transfer of these species as well as other oligomeric conformers may therefore underlie the spread of pathology and neuronal demise in PD.

that express human WT or A53T mutant α-synuclein in dopaminergic neurons have been

generated and display dopamine neuron dysfunction and loss, as well as motor deficits

(Lakso et al., 2003; Cao et al., 2005; Kuwahara et al. 2006; Cao et al., 2010). Increasing

cytosolic dopamine in WT α-synuclein-expressing worms by mutation of the VMAT2

worm homolog was found to accelerate neurodegeneration. Conversely, protec-

tion from neuronal loss was reported for worms expressing WT α-synuclein and a non-functional homolog of tyrosine hydroxylase and therefore do not synthesize dopamine (Cao et al., 2010). These studies are entirely consistent with our findings in mice that indicate dopamine and α-synuclein act together to induce neurodegeneration. To expand upon this work and test the role of a direct interaction between α-synuclein and dopamine, worms could be generated expressing human WT or A53T α-synuclein with the previously described C-terminal 125-FAAFA-129 substitution (Norris et al., 2005; Mazzulli et al., 2007). These worms would be expected to show amelioration of the neurodegenerative phenotype and resistance to the neurotoxic effects of increasing cytosolic dopamine. Moreover, an analysis of α-synuclein oligomers would be expected to correlate with disease, such that oligomer burden would be increased in α-synuclein/ VMAT2 mutants and decreased in both 125-FAAFA-129 and 125-FAAFA-129/VMAT2 mutants. To my knowledge, oligomeric α-synuclein has not been previously reported in worm models and these would therefore constitute novel biochemical findings.

4.3 Putative mechanisms of toxicity for dopamine-induced α-synuclein oligomers

Several mechanisms of α-synuclein oligomer toxicity have been proposed, including disruption of membranes, endoplasmic reticulum stress, mitochondrial dysfunction, impairment of proteasomal and autophagic degradation, and induction of neuroinflammation (Roberts and Brown, 2015). Dopamine-stabilized α-synuclein oligomers have been shown to impair chaperone-mediated autophagy and SNARE complex formation *in vitro* (Martinez-Vicente et al., 2008; Choi et al., 2013). In addition, toxic α-synuclein aggregates are thought to   propagate by seeding the conversion of

native α-synuclein into pathological conformations (Goedert et al., 2010). In Chapter 2, dopamine-modified oligomers extracted from the mouse SN were found to be incapable of seeding the aggregation of recombinant α-synuclein in an *in vitro* assay. This is in contrast to the efficient seeding activity observed for oligomers from control mice, suggesting that seeding-competent species are not retained following elevation of dopamine levels *in vivo*. These data support the notion that dopamine promotes modified conformations of oligomers, with functional consequences that may be relevant to their toxicity. Moreover, it appears that toxicity of these species is not mediated through a seeding mechanism, in contrast to previous reports from A53T mice showing that seeding-competent oligomers from the spinal cord induced neuronal injury in primary cultures whereas oligomers from the olfactory bulb that were found to inhibit aggregation were non-toxic (Tsika et al., 2010). Clearly, further study is warranted to uncover the relationship between distinct oligomeric conformations, seeding competency, and neurotoxicity.

An alternative mechanism by which dopamine-induced α-synuclein oligomers may endanger neuronal health is through the disruption of cellular membranes. Recombinant α-synuclein oligomers have been shown to bind brain-derived and synthetic phospholipid membranes and permeabilize synthetic vesicles (Volles et al., 2001; Ding et al., 2002). PD-linked A53T and A30P mutations exhibit an enhanced ability to permeabilize vesicles *in vitro* (Volles et al., 2002). In some cases, recombinant oligomers have displayed ring-like morphologies by electron microscopy and atomic force microscopy, suggesting that membrane disruption may occur through a pore-forming mechanism (Conway et al., 2000b; Lashuel et al., 2002; Ding et al., 2002).

Consistent with aberrant membrane interactions, α-synuclein oligomers (in one study, with annular morphology confirmed) have been shown to increase intracellular calcium levels and induce caspase activation and cell death when applied to primary neuronal cultures or SH-SY5Y cells (Danzer et al., 2007; Angelova et al., 2016). The ability of dopamine-induced oligomers to cause calcium dysregulation has not been explored, although calcium and α-synuclein were both required for neuronal death in midbrain cultures following an increase in cytosolic dopamine levels (Mosharov et al., 2009).

To test if recombinant α-synuclein oligomers generated in the presence of dopamine are able to elevate intracellular calcium, primary hippocampal neurons were exposed to oligomer preparations following pre-loading with Fluo-4 AM calcium-binding dye (see Appendix for detailed methods). The extracellular bath contained physiological concentrations of calcium (1 mM). Administration of a toxic dose of dopamine-incubated α-synuclein (1 μM) resulted in an approximately 3% increase in fluorescence within 5 seconds, and fluorescence remained elevated for at least an additional 15 seconds (Figure 4.2). Interestingly, this effect was observed in axons (Figure 4.2a-b) and not in cell bodies (Figure 4.2c-d). Equivalent doses of PBS or monomeric α-synuclein were incapable of producing the same response, indicating that the rise in intracellular calcium was mediated by oligomers. Furthermore, omission of extracellular calcium abolished the effect in axons, consistent with oligomer-mediated disruption of membrane integrity and calcium influx (Figure 4.2a-b). Further investigation is necessary, however, to exclude the possibility of calcium entry through voltage-gated calcium channels or glutamate receptors. These data suggest that dopamine-induced α-synuclein oligomers can interact with cellular membranes and   disturb calcium homeostasis. Entirely

consistent with our findings is prior evidence showing that seeding-incompetent α-synuclein oligomers induced calcium influx and cell death in SH-SY5Y cultures while oligomers that were capable of seeding had no effect on calcium or cell viability (Danzer et al., 2007). Follow-up experiments could examine the relationship of internalized oligomers with intracellular membranes and calcium stores.
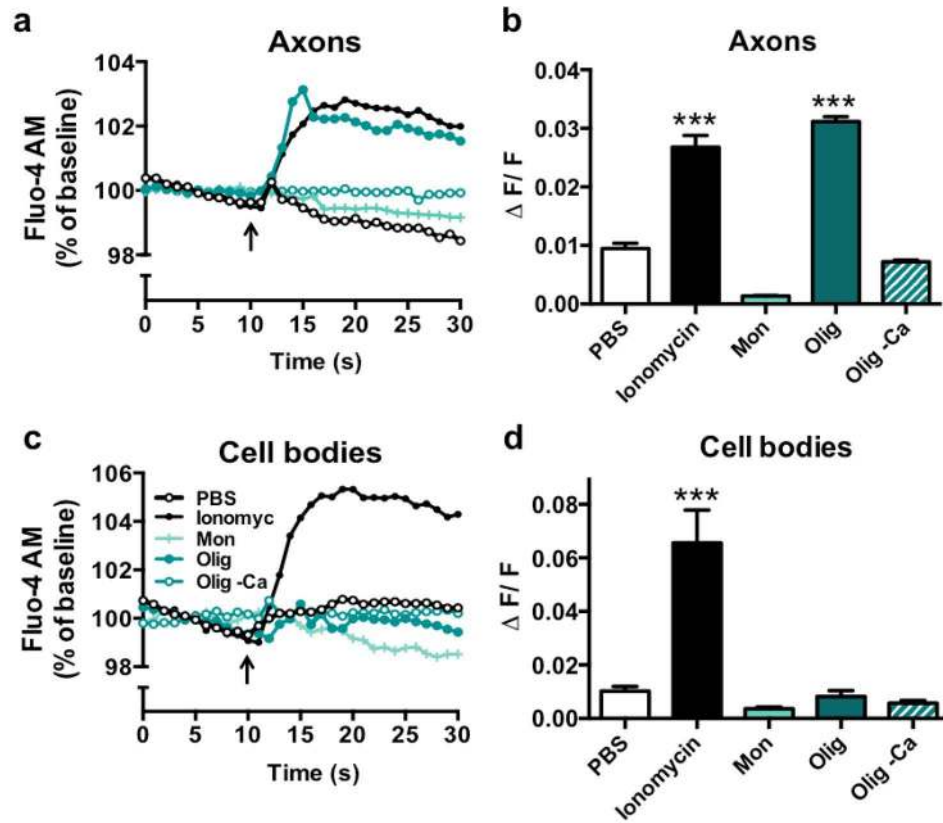
Figure 4.2: Dopamine-induced α-synuclein oligomers cause calcium dysregulation in primary neurons. Hippocampal neurons at 7 days in vitro were pre-loaded with Fluo-4 AM dye and treated with 1 μM α-synuclein oligomer (Olig) or equivalent doses of monomer (Mon), ionomycin (Ionomyc), or PBS. Representative traces of fluorescence intensity before and after the time of treatment (indicated by the arrows) are shown. Oligomers induced a significant increase in fluorescence specifically in axons **(a-b)** and not in cell bodies **(c-d)**. Exclusion of calcium from the extracellular bath (Olig -Ca) eliminated the effect of oligomers on axons, suggesting that oligomers allow calcium entry into cells. The data are presented as mean ± s.e.m. (Axons, $n = 3$ except $n = 4$ for PBS and Olig; Cell bodies, $n = 3$ except $n = 4$ for Olig and $n = 2$ for Ionomycin; one-way ANOVA with Tukey's correction for multiple comparisons). ***$P < 0.001$. DEM and HI designed the experiments, VXT collected the data, and VXT and DEM analyzed the results.

4.4 Is L-DOPA therapy toxic?

Since the discovery that striatal dopamine is severely depleted in PD patients (Ehringer and Hornykiewicz, 1960), followed immediately thereafter by the first clinical use of L-DOPA to replenish dopamine levels (Birkmayer and Hornykiewicz, 1961), L-DOPA has become the gold standard of PD treatment. Undeniably, the resulting symptomatic relief has markedly improved patients' quality of life. However, due to the notion that dopamine may be neurotoxic, there has been a widespread practice of 'DOPA sparing' that delays administration of the drug until more advanced stages of disease (Caudle et al., 2008). It is therefore of utmost importance to determine if L-DOPA has any deleterious effects, in order to inform therapeutic approaches and ensure that patients receive the best quality of care.

At present, there is no conclusive evidence indicating that L-DOPA therapy accelerates PD progression (Olanow, 2015). A number of studies have been conducted in PD patients to test the question of L-DOPA toxicity, including trials based on clinical outcome (Diamond and Markham, 1990; Fahn et al., 2004; Gray et al., 2014), neuroimaging markers (Parkinson Study Group, 2002; Whone et al., 2003; Fahn et al., 2004), and postmortem analyses (Parkkinen et al., 2011). With the exception of the neuroimaging studies, which found that L-DOPA was associated with an accelerated decline in dopaminergic markers (Parkinson Study Group, 2002; Whone et al., 2003; Fahn et al., 2004), the consensus has been that L-DOPA either does not affect PD progression or in some cases may actually be neuroprotective. However, the results of these studies are called into question by the significant limitations in their designs and methodologies. Moreover, the symptomatic relief provided by L-DOPA is likely a

confounding factor in the assessment of underlying neuronal health and disease progression. Thus, the issue of L-DOPA toxicity has unfortunately remained unresolved (Olanow, 2015).

By the time PD patients manifest motor symptoms and receive L-DOPA treatment, a significant number of dopaminergic neurons in the SN have already been lost. In addition, evidence from PD brains suggests that dopaminergic terminals succumb to disease even earlier than neuronal cell bodies (Cheng et al., 2010), as is also the case in our mouse model, indicating that much of the disease process has occurred long before L-DOPA therapy is initiated. While a role for L-DOPA in advancing neurodegeneration cannot be ruled out, my findings are instead more relevant to the etiology of PD, pointing to a potential mechanism for the initial demise of dopaminergic neurons involving the aberrant interaction of oxidized dopamine with α-synuclein.

4.5 Concluding remarks

A unifying theory of neurodegeneration in PD must account for several features of the disease: the loss of dopaminergic neurons in the SN and the relative sparing of dopaminergic neurons in the neighboring ventral tegmental area (VTA), the loss of non-dopaminergic cells in other brain regions such as the dorsal motor nucleus of the vagus and the nucleus basalis of Meynert, and the presence of α-synuclein-positive inclusions in both degenerating and non-degenerating neuronal populations. The work presented in this thesis offers clues as to the disease mechanisms that may give rise to these features, and points to novel directions for future research.

As proposed in Chapter 3, PD may begin when catastrophic events occur allowing native α-synuclein to become aggregation-competent. These could include age-related deterioration of proteostasis pathways, such as proper folding of α-synuclein by chaperones or degradation of improper conformations (Balch et al., 2008). Following escape from protein quality control mechanisms, α-synuclein may aggregate into oligomers and fibrils, eventually forming Lewy body inclusions and Lewy neurites. This may occur independently in different brain regions, or as growing evidence suggests, may arise through prion-like transmission of α-synuclein across synaptic connections (Goedert et al., 2010). Different oligomer species can have different levels of toxicity (Danzer et al., 2007; Tsika et al., 2010), and region-specific factors may determine whether toxic oligomers are able to form and induce neurodegeneration. In catecholaminergic regions such as the locus coeruleus and SN, oxidized cytosolic dopamine may act on oligomers and/or monomers resulting in particularly damaging oligomeric conformations. These species may then induce cell death potentially through aberrant membrane interactions and disruption of calcium homeostasis. SN neurons may be more susceptible to calcium dysregulation than VTA neurons due to their dependence on calcium for pacemaking and lower intrinsic calcium buffering capacity (Surmeier et al., 2013).

Overall, this thesis provides four novel findings: First, the toxicity of a sustained elevation in striatal dopamine was tested for the first time, and surprisingly, was insufficient to produce dopaminergic cell death. Second, and in stark contrast, raising dopamine levels in the context of higher α-synuclein expression resulted in progressive nigrostriatal degeneration and a locomotor deficit. Third, for the first time *in vivo*, dopamine was shown to promote α- synuclein oligomerization yielding

species with unique conformations and activity. Lastly, calcium dysregulation was implicated as a potential mechanism of dopamine-modified oligomer toxicity. α-Synuclein oligomers arising from the pathological interaction with dopamine may be an important new target in the design of disease-modifying therapies.

APPENDIX

Materials and Methods

Measurement of intracellular calcium was performed as previously described (Danzer et al., 2007). Briefly, following one week in culture, cells were washed with Ringer buffer containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM glucose, and 20 mM HEPES. Cells were loaded for 60 min with 2 µM cell-permeable Fluo-4 AM (Thermo Fisher) in Ringer buffer, then washed with Ringer buffer, incubated at RT for 30 min, and washed again. Cells were treated with 1 µM dopamine-incubated α-synuclein (from day 4-6 of *in vitro* aggregation) or control treatments. For each well, fluorescence imaging was conducted in the same field of view for 30 seconds prior to treatment and for up to 5 min total. Intensity measurements were obtained with MetaMorph software (Molecular Devices) and the baseline was calculated as the average intensity in the 10 seconds prior to treatment. The ΔF/F was calculated as (maximum peak height - baseline) / baseline. To determine calcium source, calcium was excluded from the Ringer buffer and experiments were performed as described above.

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