

Investigating the mechanisms underlying synaptic and cognitive deficits in alpha-synucleinopathies

A Dissertation

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ABSTRACT

Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB) are clinically and neuropathologically highly related α -synucleinopathies that collectively constitute the second leading cause of neurodegenerative dementias. Genetic and neuropathological studies directly implicate α -synuclein (α S) abnormalities in PDD and DLB pathogenesis. However, it is currently unknown how α S abnormalities contribute to memory loss, particularly since forebrain and hippocampal neuron loss in PDD and DLB is less severe than in Alzheimer's disease.

Previously, we found that familial Parkinson's disease (PD)-linked human mutant A53T α S causes aberrant localization of the microtubule-associated protein tau to postsynaptic spines in neurons, leading to postsynaptic deficits. Thus, we directly tested if the synaptic and memory deficits in a mouse model of α -synucleinopathy (TgA53T) are mediated by tau. TgA53T mice exhibit progressive memory deficits associated with postsynaptic deficits in the absence of obvious neuropathological and neurodegenerative changes in the hippocampus. Significantly, removal of endogenous mouse tau expression in TgA53T mice (TgA53T/mTau^{-/-}), achieved by mating TgA53T mice to mouse tau knockout mice, completely ameliorates cognitive dysfunction and concurrent synaptic deficits without affecting α S expression or accumulation of selected toxic α S oligomers. Among the known tau-dependent effects, memory deficits in TgA53T mice were associated with hippocampal circuit remodeling linked to chronic network hyperexcitability. This remodeling was absent in TgA53T/mTau^{-/-} mice, indicating that postsynaptic deficits, aberrant network hyperactivity, and memory deficits are mechanistically linked. Our results directly implicate tau as a mediator of specific human mutant A53T α S-mediated abnormalities related to deficits in hippocampal neurotransmission and suggest a mechanism for memory impairment that occurs as a consequence of synaptic dysfunction

rather than synaptic or neuronal loss. We hypothesize that these initial synaptic deficits contribute to network hyperexcitability which, in turn, exacerbate cognitive dysfunction.

α S is also central to sporadic PD pathogenesis. Pathogenic species of α S, such as fibrils, have recently been implicated as a pathogenic component of synucleinopathies, capable of transmission between functionally and anatomically connected brain regions, including the hippocampus. However, how α S fibrils impact hippocampal function and contribute to memory deficits is not well understood. We hypothesized that α S fibril-induced synaptic changes could be mediated by other through interactions with other proteins, including tau. Wild-type primary hippocampal neurons acutely exposed to fibrillar α S species display tau missorting to dendritic spines as well as electrophysiological deficits in both pre and postsynaptic neurotransmission. However, some of these findings may be a product of concentration-dependent α S fibril-induced spine collapse. Importantly, α S fibril-mediated tau missorting and synapse loss could be differentiated by examining key proteins and pathways *in vitro*, pointing to both structural and functional deficits at hippocampal synapses as contributors to cognitive impairment in PDD and DLB. Taken together, these initial studies using α S fibrils suggest that pathologic species of α S may act through distinct intracellular and extracellular mechanisms to contribute to neuronal dysfunction and neuronal toxicity.

Through these two approaches and methodologies, our results indicate that these synaptic changes, along with key pathological species of α S, present potential therapeutic targets for amelioration of memory deficits in α -synucleinopathies.

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ABBREVIATIONS

α -amino-3-hydroxy-methyl-4-isoxazolepropionic acid	AMPA
α -synuclein	α S
Alzheimer's disease	AD
AMPA receptor (AMPA) subunit	GluA_
Cortex	CTX
Days <i>in vitro</i>	DIV
Dementia with Lewy bodies	DLB
Enhanced green fluorescent protein	eGFP
Excitatory postsynaptic current	EPSC
Hippocampus	HIP
Human α -synuclein	h α S
Human missense mutant A53T α -synuclein	h α S ^{A53T}
Human wild-type α -synuclein	h α S ^{WT}
Human wild-type α -synuclein pre-formed fibrils	α S PFFs
Human tau	hTau
Lewy body	LB
Lewy neurite	LN
Long-term potentiation	LTP
Mini excitatory postsynaptic current	mEPSC
Month	M
Mouse tau	mTau
N-methyl-D-aspartate	NMDA
NMDA receptor (NMDAR) subunit	GluN_
Non-transgenic	nTg
Parkinson's disease	PD
Parkinson's disease dementia	PDD
Pre-formed fibrils	PFFs
Substantia nigra pars compacta	SNPc
Wild-type	WT

INTRODUCTION

Non-communicable diseases are on the rise and neurological disorders are leading the charge

Over history, disease burden has shifted. Within the last century, while the epidemiological burden of infectious diseases (e.g. smallpox and polio) as well as chronic and developmental diseases (e.g. nutritional deficiencies) has lessened, others have risen. Initiated in the 1990s by the World Bank, the Global Burden of Diseases (GBD), now organized by the World Health Organization, has brought global attention to hidden and neglected health challenges, as well as revealed alarming trends. One such trend points to a shift in the types of diseases affecting the world: as deaths from communicable diseases decreased in 2016, more than three-quarters of all deaths in 2016 were caused by non-communicable diseases (NCDs) (James et al., 2018). Currently, NCDs now account for more than one-half of the global burden of disease. Compounding this increasing burden of NCDs is the growing global population. By 2050, the global population aged 60 and 80 years or older is expected to double and triple, respectively (United Nations, 2017 World Population Aging Study). This increase in the aging population is accompanied by an increase in diseases that are more prominent in later stages of life. In particular, neurological disorders are NCDs and major contributors to the global disease burden as the second leading cause of death in 2015 and leading cause of disability worldwide (Feigin et al., 2019). The number of people living with Alzheimer's disease (AD) and other dementias are among the most burdensome of neurodegenerative diseases worldwide and has increased over 100% since 1990. In 2016, dementia was the fifth leading cause of death globally (Nichols et al., 2019). In that same time frame, the number of people with Parkinson's disease (PD), the second most common neurodegenerative disorder, has also more than doubled. PD is now the fastest growing of any neurological disease, seeing the greatest rise in prevalence, disability, and mortality

(Dorsey et al., 2018). A conservative projected doubling of patients over the next 30 years would yield more than 12 million PD patients by 2050.

Within the United States, the economic burden of PD exceeded \$20 billion in 2010 (Kowal et al., 2013), a value that will most certainly increase with an expanding PD population. Moreover, almost 60% of the \$14 billion in direct medical costs due to PD is associated with nursing home services, highlighting the strain an aging and diseased population places not only on the patient, but also medical providers and caregivers. The economic cost of PD is substantial, and increases as the disease progresses. While a curative silver bullet for PD is desired and a valid motivator for current research, the need for preventative and disease-modifying therapies are critical in combatting the complex burden an aging population presents. Interestingly, one model estimated that a treatment that slows PD progression by 50% would yield a 35% reduction in costs, a dramatic reduction in cost of care that is coupled to a longer expected survival (Johnson et al., 2013).

Unfortunately, no effective curative or disease-modifying treatments exist for these major neurodegenerative diseases and causes of dementia. There is an emerging need to bridge the gap between life expectancy and healthy life expectancy. Until therapeutic breakthroughs are made, neurological disorders, neurodegenerative disease and dementia in particular, will constitute an increasing challenge to health-care systems worldwide. To that end, there is an urgent need for research identifying new interventions for people with PD.

Parkinson's disease is a rising neurological disorder

The increasing contributions neurodegenerative diseases, including PD, impart on the global burden of disease supports placing them on the top of the global public health agenda. The dramatic rise in PD's prevalence, disability, and deaths can partly be

explained by a growing global population as well as increased longevity. Age is the greatest risk factor for PD, affecting 1% of the population of 60 years of age, and 5% of the population over the age of 85 (Van Den Eeden, 2003). PD is rare before 50 years of age, but the incidence increases 5-10-fold from the sixth to ninth decade of life (Poewe et al., 2017). The progressive increase in the number of aged individuals is one driver of an increase in PD prevalence, but the incidence of PD has also increased since 1976, particularly in men 70 years or older (Savica et al., 2016). Unfortunately for us, age is an unmodifiable risk factor. Given the lack of preventative, disease-modifying, or curative treatments for PD, identification of modifiable risk factors is critical to understanding key drivers of these trends.

Environmental and social factors have consequently come into the limelight. Industrial and chemical and pollutants, such as pesticides, solvents, and metals appear to be associated with an increased risk for PD (Pezzoli and Cereda, 2013; Tanner et al., 2011; Weisskopf et al., 2010). Surprisingly, there is a lower risk of Parkinson's disease among smokers, both current and former (Thacker et al., 2007). Dramatic changes in smoking behavior that include increased cessation and decreased use (Dorsey et al., 2018; Savica et al., 2016) may partly explain the rise of PD over the past several decades, but a mechanistic understanding of this relationship remains unknown.

In addition to population aging, another emerging trend in global demographics is urbanization. In 2018, 55% of the world lived in urban areas, but is expected to increase to 68% by 2050 (United Nations, 2018 Revision of World Urbanization Prospects). Paralleling the idea of environmental exposures as disease risk factors, ambient air pollution from traffic sources is associated with an increased risk for PD (Ritz Beate et al., 2016). Interestingly, a higher incidence of neurodegenerative diseases and cognitive dysfunction has been observed in individuals living in close proximity to major roads (Chen et al., 2017; Wellenius et al., 2012; Wilker et al., 2015). Taken together, managing the

health of an aging, more urbanized global population presents quite a challenge. Complementing our need to better identify risk factors driving neurological disease and PD in particular, a better understanding of the key mechanisms driving PD pathogenesis are critical to improving disease management and quality of life for those affected by PD.

Parkinson's disease: a brief history

PD was first medically described as a collective, single neurological syndrome by British physician James Parkinson in 1817 in his "Essay on the Shaking Palsy" (Parkinson, 2002). In his "Essay," Parkinson systematically outlined a syndrome he had observed in six patients in London, discussing the progressive and disabling condition, parkinsonian posture, festinant (Parkinsonian) gait, and tremor. While these clinical symptoms had been observed before, going as far back at Galen during the Roman Empire, their understanding was limited and classification into a common disorder was incomplete. Dutch physician Fanciscus Sylvius first described resting tremor in 1680. And François Boissier de Sauvages, a French physician, wrote on the Parkinsonian gait in 1768. Moreover, prior to Parkinson's essay, using tremors alone as diagnostic criteria meant significant overlap with other conditions such as seizures.

In 1872, Jean-Martin Charcot, a French neurologist and largely considered to be the founder of modern neurology, advanced Parkinson's clinical observations. Charcot was instrumental in recognizing that patients, similar to the cohort described by Parkinson, all displayed tremor, rigidity, facial immobility, postural instability, and bradykinesia. With this new understanding of the cardinal motor features, Charcot was the first to suggest the term "Parkinson's disease" to define this constellation of symptoms (Goetz, 1986). Prior to Charcot, the classification of neurological diseases was primitive, where disorders were grouped by primary symptoms, such as tremors or weakness. Having established the

symptoms, Charcot now had tools in place to classify PD as a neurological disorder and differentiate it from other tremor-producing disorders such as multiple sclerosis.

Fritz Heinrich Lewy, in 1912, was the first to describe characteristic proteinaceous intra-neuronal inclusions bodies in several brain regions of patients with PD, including the dorsal motor nucleus of the vagus nerve. Lewy's work was shortly followed by that of Konstantin Tretiakoff in 1919. Tretiakoff's anatomical and pathological studies identified a severe loss of pigmented neurons in the substantia nigra in PD patients. In surviving nigral cells, he also reported protein aggregates, subsequently terming them "Lewy bodies" (Goedert et al., 2013).

Insight into substantia nigra (SN) dysfunction and PD came from Swedish scientist Arvid Carlsson. His group first noticed drug-induced parkinsonism in rabbits treated with the dopamine antagonist, reserpine, and subsequently identified dopamine's presence in the brain (Carlsson et al., 1958). Having demonstrated high dopamine concentrations in the striatum, Carlsson was the first to propose dopamine as a neurotransmitter, acted on circuits involved in motor control, and deficiency of this catecholamine might underlie the motor symptoms in PD (Carlsson, 2002; Lees et al., 2015). Austrian biochemist Oleh Hornykiewicz shortly after advanced Carlsson's hypotheses by documenting dopamine deficiency in the striatum and SN in individuals with PD (Lees et al., 2015). Californian neurologist William Langston's clinical and pathological study of sudden-onset parkinsonism in patients with prior history of intravenous drug use aged 26 to 42 years yielded two major benefits: 1) further implicating cell death in the SN and Lewy pathology as findings specific for parkinsonism, and 2) identifying 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP, "synthetic heroin") as the compound injected into these patients suggested that PD could be caused by environmental factors (Langston et al., 1983). The remarkable selectivity of MPTP for producing neurotoxicity in the SN has since proved invaluable in both *in vitro* and *in vivo* toxin models of PD. Collectively, these

findings provided the initial evidence connecting the substantia nigra, dopaminergic neurotransmission, and PD together that has since guided a century of research on PD pathogenesis and therapy.

In light of progressive advances into the neuroanatomical, pathological, and neurochemical basis for PD, the causes driving these changes in the disease remained unknown for almost two centuries following Parkinson's "Essay." The first piece of evidence linking PD to the presynaptic neuronal protein α -synuclein (α S) came from the first study demonstrating a genetic abnormality in the α S gene as casual for PD. This single nucleotide G209A mutation in the *SNCA* gene lead to the missense A53T amino acid substitution identified in an Italian kindred and several Greek families with an autosomal dominant pattern (Polymeropoulos et al., 1997). An interesting caveat to this finding came from the finding wild-type (WT) rodent *SNCA* homolog sequence that has threonine (T) at amino acid residue 53 (though this is just one of seven total amino acids that differ between rodent and human α S) (Hamilton, 2004). This familial mutation raised the questions if rodents, such as mice (*Mus musculus*) and rats (*Rattus rattus*) used in the laboratory setting, were more likely to develop parkinsonism. Identification of the A30P and E46K missense point mutations in *SNCA* for autosomal dominant PD in distinct families soon followed, further highlighting a genetic component in the development of PD (Krüger et al., 1998; Zarranz et al., 2004). More recent studies identifying the G51D and H50Q missense mutations have further pointed to mutant α S a strong genetic factor in familial PD (Appel-Cresswell et al., 2013; Proukakis et al., 2013). Interestingly, all these PD-linked point mutations are located within the amino terminal (N-terminal) domain, suggesting that interference in membrane interactions mediated by this region may be one pathogenic mechanism underlying PD pathophysiology. The connection between *SNCA*, α S, and PD was further strengthened through subsequent studies demonstrating

that duplications or triplications in the WT human *SNCA* gene were sufficient to cause familial autosomal dominant PD (Chartier-Harlin et al., 2004; Singleton et al., 2003). These findings led to the hypothesis and subsequent observation that increased α S in the brain could be a potential contributor to PD pathogenesis (Miller et al., 2004).

Although familial forms only account for a small percentage of all PD cases, identifying a genetic link from *SNCA* to PD advanced the understanding of disease onset and pathogenesis. Through the use of newly-generated antibodies against α S, it was shown that Lewy bodies (LBs) and Lewy neurites (LNs) in the substantia nigra of idiopathic PD patients, along with several dementia with Lewy body (DLB) cases, immunostained for α S (Spillantini et al., 1997). The use of these α S antibodies not only helped to define the protein components of Lewy pathology, but simultaneously provided insight into the filamentous organization of α S in LBs and LNs (Spillantini et al., 1998a). With *SNCA* gene duplications, triplications, or mutations being causal for PD, and α S comprising the major protein component of PD (Lewy) pathology in both familial and sporadic PD cases, it is evident that the mechanisms underlying neurodegenerative changes in PD is likely to involve α S.

α -Synuclein is a major component of Parkinson's disease pathophysiology

α S is a 140 amino acid abundantly expressed protein throughout the nervous system that comprises upwards of 1% of total cytosolic protein. Although now synonymous with PD, the discovery of α S in normal neurophysiology began with its identification in both nuclei and presynaptic nerve terminals in the electric organ of the Pacific electric ray (*Torpedo californica*) by using antibodies against purified synaptic proteins (Maroteaux et al., 1988). α S, along with its 134 amino acid β -synuclein (β S) homolog, were subsequently identified in rat brains (Maroteaux and Scheller, 1991), and shortly after, human

hippocampi (Jakes et al., 1994). While amino acid residues 61-95 of α S, now termed the “non-A β component of AD” (NAC) domain, were also discovered as components of amyloid- β (A β) plaques (Ueda et al., 1993), this finding has failed to be confirmed (Bayer et al., 1999). However, these cross-species similarities provided for the establishment of the homologous synuclein protein family in humans that includes α S, β S, and γ -synuclein (γ S), all three of which are predominantly neuronal proteins that preferentially localize to presynaptic terminals under physiologic conditions (Stefanis, 2012).

α S is encoded for by the *SNCA* gene, located on the long arm of chromosome 4: 4q22.1. α S primarily exists in the brain as a monomer (Burré et al., 2013), but its assembly into higher order structures, such as oligomers or tetramers under both physiological and pathologic conditions, while controversial, has been demonstrated (Bartels et al., 2011; Burré et al., 2014; Nuber et al., 2018). Still, although defined as a “natively unfolded” or “intrinsically disordered” protein, α S does adopt an α -helical secondary structure upon binding to negatively charged lipids, such as phospholipid head groups present on cellular membranes. The protein can be divided into three regions: a highly conserved N-terminal repeat region, hydrophobic middle, and negatively-charged carboxy-terminal (C-terminal) region. The N-terminus contains (residues 7-87) seven repeats of an 11 amino acid residue sequence, each having a conserved six acid sequence core of KTKEGV. Although isolated alongside presynaptic vesicles, α S is located adjacent to, but not inside these structures (Maroteaux et al., 1988). α S prefers membranes with high curvature. This biological penchant for presynaptic terminals and synaptic vesicles is likely mediated through its N-terminus amino acid repeat sequences adopting an α -helical structure upon contact with such membranes (Chandra et al., 2003; Fortin et al., 2004; Jensen et al., 1998). α S is unique as the only synuclein to harbor the hydrophobic NAC domain, potentially contributing to its unique aggregation-prone status when compared to β S or γ S

(Conway et al., 2000a; Weinreb et al., 1996). The acidic C-terminus is polar, mostly unstructured, and contains several phosphorylation sites including serine at residue 129 (pSer129) (Bendor et al., 2013; Samuel et al., 2016). Phosphorylation of α S, and in particular Ser129 serves to regulate its structure, membrane binding, protein interactions, multimer organization, and neurotoxicity (Canerina-Amaro et al., 2019; Chen and Feany, 2005; Samuel et al., 2016).

In addition to phosphorylation, α S is subject to other forms of post-translational modifications. N-terminal acetylation of α S is observed in both healthy and individuals with PD, and data supports a role for acetylation increasing membrane affinity and aggregation resistance (Dikiy and Eliezer, 2014; Kang et al., 2012; Trexler and Rhoades, 2012). However, other modifications exist, such as ubiquitination (Nonaka et al., 2005), sumoylation (Krumova et al., 2011), and nitration (Giasson et al., 2000). Interestingly, monoubiquitinated forms of α S are aggregate-prone and more likely to be components of LBs as compared to being targeted for proteasome-mediated degradation (Rott et al., 2008). Proteolysis of α S, and in particular C-terminal truncation, has been demonstrated to be pathogenic (Li et al., 2005; Murray et al., 2003).

The function of α -Synuclein

The investigation of α S function predates its implication in disease. The presynaptic localization of α S was first supported by studies showing isolation of the protein alongside purified cholinergic vesicles, suggesting to a presynaptic role for the protein (Maroteaux and Scheller, 1991). In brain regions of the zebra finch uniquely involved in bird song acquisition, sustained reductions in α S expression were observed, suggesting a role for the protein in synaptic plasticity (George et al., 1995). Despite this, several lines of evidence point to α S not being critical for synaptic or nervous system function. First, α S

expression is restricted to vertebrates, and homologs in other laboratory model organisms such as yeast, worms, or flies have not been identified (George, 2002). Second, α S appears to arrive at developing synapses only after integral synaptic and membrane proteins have entered the region (Withers et al., 1997). And lastly, mice lacking α S expression (*SNCA*^{-/-}) are viable, fertile, display intact gross nervous system and neuronal architecture including dopaminergic neurons, fibers, and synapses, with only subtle alterations in striatal DAergic neurotransmission being noted (Abeliovich et al., 2000). As such, it is likely that α S contributes to more complex neuronal activities beyond synaptic development or basic neurotransmitter release.

At the synapse, α S exists between soluble or membrane-bound states. Although soluble, cytosolic α S behaves as a natively unfolded protein (intrinsically disordered protein) (Binolfi et al., 2012; Fauvet et al., 2012; Weinreb et al., 1996). However, there is evidence to suggest α S may exist as a stable tetramer in physiological conditions that protects against aggregation and breaks down in pathological states like PD (Bartels et al., 2011; Nuber et al., 2018; Wang et al., 2011). α S binding to lipid-rich surfaces, such as cell membranes and synaptic vesicles, can induce α S stabilization and aggregation into multimeric species (Burré et al., 2014). This process may be due to the N-terminal portion of α S adopting an α -helical structure in the presence of lipids, which further mediates its binding to membranes (Bussell and Eliezer, 2003; Middleton and Rhoades, 2010). In contrast, under pathological states, α S adopts a β -sheet-rich conformation that promotes aggregation, fibrillization, and deposition into pathological Lewy structures (Conway et al., 1998, 2000b)

It is hypothesized that α S localizes to presynaptic terminals due to its preference for highly curved membranes (Maroteaux et al., 1988; Nuscher et al., 2004). As synaptic vesicle membranes represent some of the smallest curved membranes within a neuron,

this, in combination with α S's affinity for vesicular SNARE proteins, synapsins, and Rab3A likely aid in its targeting to the presynaptic site (Chen et al., 2013; Kahle et al., 2000; Zaltieri et al., 2015). Consistent with this, α S disperses from presynaptic boutons following stimulation, suggesting that the protein may dissociate from the vesicle membrane when it fuses with the presynaptic terminal plasma membrane and flattens out (Fortin et al., 2005). Another driving force behind the presynaptic localization of α S may be its colocalization with lipid rafts, regions of plasma membrane enriched in cholesterol and saturated acyl chains, often found at the presynaptic bouton (Fortin et al., 2004).

The presynaptic location of α S and its preference for synaptic proteins and vesicles has guided efforts to understand α S function in the context of presynaptic neurotransmission. Initial work in the presynaptic context proposed a role for α S in promoting vesicle release, suggesting that *SNCA*^{-/-} mice have an impaired capacity to activate reserve pools of vesicles following prolonged stimulation (Cabin et al., 2002). However, this contrasts more contemporary studies demonstrating a mild reduction in DA content in striatal neurons, consistent with increased DA release in *SNCA*^{-/-} mice (Abeliovich et al., 2000). DA release in neurons from mice lacking α S also shows more rapid facilitation and attenuated depression following repeated stimulation (Yavich et al., 2004), suggesting a role for α S in regulating vesicle mobilization as a means to modulate neurotransmission.

As increased α S expression is associated with, and causal for, PD (Miller et al., 2004; Singleton et al., 2003), it has also been valuable to study the consequences of α S overexpression on synaptic physiology. Overexpression of human wild-type α S (h α S^{WT}) in the PC12 chromaffin cell line has been found to inhibit dense core vesicle exocytosis as measured by a reduction in total events without affecting key synaptic aspects like quantal size (Larsen et al., 2006). Another study showed similar findings in primary

midbrain neurons, primary hippocampal neuron, and *ex vivo* slices in recording from CA1 pyramidal neurons, where α S overexpression reduced synaptic vesicle exocytosis without affecting quantal size or production of overt neurotoxicity (Nemani et al., 2010). Interestingly, while $h\alpha S^{WT}$ overexpression in PC12 cell studies also revealed an accumulation of granules adjacent to the plasma membrane (Larsen et al., 2006), and $h\alpha S^{WT}$ overexpression in hippocampal neurons displayed reduced synaptic vesicle recycling dynamics, with vesicles accumulating in regions away from the synapse active zone (Nemani et al., 2010). A disease-relevant caveat to α S-mediated inhibition of exocytosis is the A30P missense mutation associated with familial PD (Kruger et al., 2001). Human A30P α S ($h\alpha S^{A30P}$) has been shown to impair α S interactions with membranes (Jo et al., 2002). However, while overexpression of $h\alpha S^{A30P}$ in neurons does not produce inhibition of exocytosis as compared to its WT counterpart (Nemani et al., 2010), PC12 cells overexpressing either $h\alpha S^{WT}$ or $h\alpha S^{A30P}$ had similar effects on decrease exocytotic function (Larsen et al., 2006). Taken together, these studies demonstrate that α S functions in the modulation of neurotransmission in several neuronal types, including dopaminergic and glutamatergic neurons.

The regulatory aspect of α S may also be due to its ability to form multimers following membrane interaction (Burré et al., 2014), whereby α S reportedly clusters synaptic vesicles in neurons away from the active site to further regulate vesicle mobilization and exocytosis (Wang et al., 2014). This is in line with observations in neurons lacking α S that highlight a specific deficiency in vesicles not docked at presynaptic sites (Cabin et al., 2002). α S may also exert its modulatory effect on signaling through another vesicle-centric aspect, such as recycling and endocytosis of vesicles following periods of high frequency stimulation and neurotransmission (Vargas et al., 2014). These studies collectively suggest that α S suppresses synaptic transmission through vesicle exocytosis and

recycling as opposed to affecting the actual packaging of initial vesicles at presynaptic boutons. Moreover, it is likely that α S is not vital for synaptic formation or basal neurotransmission but may play a more intricate role in modulation of synaptic plasticity and maintenance of synapses.

From a biochemical perspective, α S overexpression reduces both synapsin and complexin levels, key presynaptic proteins involved in vesicle release (Nemani et al., 2010). However, homozygous knockout mice of α S (*SNCA*^{-/-}) and β S (*SNCB*^{-/-}) display restored complexin levels (Chandra et al., 2004), suggesting that pathologic levels of α S that reduce synaptic transmission do so potentially through direct mechanisms such as blocking vesicle fusion or recycling, but also indirect mechanisms, such as reducing the levels of protein machinery required for presynaptic vesicle release.

In addition to its role in modulation of neurotransmission, α S may also function to maintain presynaptic terminals. Knockout of cysteine string protein α (CSP α), a presynaptic chaperone, in mice, leads to rapid and progressive synaptic degeneration and death. However, overexpression of h α S^{WT} compensates for this loss and delays the degenerative and lethal phenotype observed in CSP α knockout mice (Chandra et al., 2005), suggesting that α S may have additional roles as a chaperone for presynaptic machinery and vesicles. Evidence supporting this hypothesis comes from another study that demonstrated that α S can directly interact with synaptobrevin 2, a member of the vesicle-SNARE (soluble NSF attachment protein receptor) complex, and potentially contribute to SNARE complex formation that mediates vesicle binding to presynaptic nerve terminals (Burré et al., 2010). This supportive role of α S in neuronal function is furthered by studies demonstrating that mice lacking all three synucleins (α S, β S, and γ S) have reduced SNARE complex formation, reduced excitatory presynaptic terminal size, neuronal dysfunction, and shortened lifespan (Anwar et al., 2011; Greten-Harrison et al.,

2010; Vargas et al., 2017). *SNCA*^{-/-} mice have also been reported to have impaired performance on behavioral assays of spatial learning and memory (Kokhan et al., 2012), suggesting that the role of α S in presynaptic neurotransmission has greater implications on synaptic plasticity and behavior.

In line with its presynaptic enrichment, α S is almost exclusively localized to neuronal tissues (Iwai, 2000). However, one exception to this is α S expression in cells of hematopoietic lineages, including red blood cells as well as platelets (Barbour et al., 2008; Li et al., 2002). Interestingly, the role of α S in the vasculature seems to parallel its role in synaptic function in the nervous system. For example, α S has been demonstrated to play a role in inhibition of granule release from platelets, serving to regulate and modulate clotting, and overexpression of familial PD-linked A53T or A30P α S mutants further reduced exocytosis of granule contents from platelets. (Park et al., 2002). PD patients, both familial and idiopathic cases, are at an increased risk for stroke, potentially shedding light onto a new mechanisms underlying PD/DLB pathogenesis, motor and non-motor symptoms, and contributors to morbidity and mortality following diagnosis of α -synucleinopathy (Garcia-Gracia et al., 2013; Jellinger, 2003).

Parkinson's disease as a movement disorder and α -synucleinopathy

Movement disorders represent a broad family of neurological diseases that often have similar clinical presentations but with strikingly different origins. As such, a clinical challenge in the diagnosis of movement disorders is understanding drivers of the presenting symptoms and differentiating among diseases with common presentations. Clinically, these disorders are classified as hyperkinetic, hypokinetic, or dystonic. Hypokinetic disorders include PD, multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), all of which can give rise

to a constellation of physical exam findings (resting tremor, bradykinesia, rigidity, postural instability) collectively termed “parkinsonism” (Stoessel and Mckeown, 2016). MSA, PSP, and CBD are also part of group of neurodegenerative diseases called Parkinson-plus syndromes, all of which possess key features to help differentiate them from each other and from PD. For example, while individuals with MSA-induced parkinsonism can display a reduction in motor symptoms in response to L-DOPA treatment, this response wanes with disease progression. PSP patients display a supranuclear gaze palsy and disease-specific midbrain findings upon MRI, both of which serve as exclusionary criteria for a diagnosis of PD as the cause of parkinsonism (Postuma et al., 2015). Further differentiation can be made pathologically as CBD and PSP are tauopathies, displaying characteristic aggregates of the protein tau in glial cells, astrocytes. In addition to being movement disorders sharing parkinsonism as common clinical findings, PD and MSA, along with DLB, also share pathological findings that place them into the synucleinopathies, a group of neurodegenerative disease characterized by the formation of intracellular α -synuclein-containing inclusions in the brain. These include Lewy bodies (LBs) and Lewy neurites (LNs) found in the neurons of PD and DLB, and glial cytoplasmic inclusions (GCIs) within oligodendrocytes of MSA patients (Yang and Yu, 2017).

Despite sharing many clinical and pathological features, cognitive deficits in particular, dementia in PD (PDD) and DLB are classified as separate entities in the latest version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Interestingly, their differential diagnosis is made based upon an arbitrary distinction concerning the temporal onset of motor and cognitive deficits. DLB is diagnosed when the onset of dementia precedes onset of parkinsonian signs for at least one year (McKeith et al., 2017). Conversely, the diagnosis of PDD is made when cognitive impairment develops in the setting of long-standing PD (Emre et al., 2007). However, the rationale for considering PDD and DLB as separate but related neurodegenerative diseases may be becoming

increasingly antiquated, and whether these two are the same disease or not has been the subject of recent debate (Friedman, 2018; Jellinger, 2018; Jellinger and Korczyn, 2018; McKeith et al., 2017). Beyond the defined 1-year rule, PDD and DLB share many non-motor symptoms, including autonomic dysfunction and psychiatric changes, further complicating clinical presentation as the singular means for differential diagnosis. As synucleinopathies, PDD and DLB share neuropathological features that include striatal LB pathology, but also cortical LB pathology (Braak Stage 5-6, Braak et al., 2003) as well as hippocampal α S pathology. It is possible that the extent of AD pathology measured either postmortem or antemortem via PET, including extracellular A β plaques and intraneuronal fibrillary tau tangles (NFTs), may represent a key pathological difference as DLB brains display higher cortical A β and tau loads (Jellinger and Korczyn, 2018). Despite this considerable pathological and clinical overlap between PDD and DLB, biomarker and neuroimaging studies have proven beneficial in providing potential mechanisms for discrimination. Elevated tau and reduced A β levels in the cerebrospinal fluid (CSF), a profile associated with AD, is higher in DLB as compared to PDD (van Steenoven et al., 2016; Vranová et al., 2014). However, unlike AD, medial temporal lobe and hippocampal volume is commonly preserved in PDD and DLB (McKeith et al., 2017). When combined as neurological disorders with shared clinical and pathological findings, PDD and DLB collectively represent the second-leading cause of dementia, behind AD (Aarsland, 2016). As such, considering them, with some caution, as a common neurodegenerative disorder is beneficial in improving our understanding of the pathogenesis and pathophysiology in α -synuclein-mediated cognitive decline.

Motor symptoms in Parkinson's disease and dementia with Lewy bodies

200 years have passed since James Parkinson's original account, but the majority of the clinical features of the eponymous disorder he described have persisted (Parkinson, 2002). Today the clinical diagnosis of PD still centers on a defined motor syndrome, "parkinsonism", defined as bradykinesia, in combination with either a resting tremor, rigidity, or both (Postuma et al., 2015; Tolosa et al., 2006). Bradykinesia is defined as slowness of movement and decreased amplitude or speed as movements are continued. A resting tremor refers to a 4- to 6-hertz (Hz) tremor in a limb at rest, which is suppressed with movement, and often described as "pill-rolling." Rigidity is observed to be a resistance to slow, passive movement of major joints when limbs are being manipulated on physical examination. Colloquially this can be referred to "cogwheel rigidity." In addition to an initial diagnosis of parkinsonism, there are additional supportive and exclusion criteria to determine whether a patient meets the criteria for PD as the cause of parkinsonism (Postuma et al., 2015). These core clinical findings, in combination with the neurodegenerative, pathologic, and dopaminergic changes, have traditionally defined PD as a motor disorder. This has, consequently, focused more basic science approaches through a motor-centric lens, pushing towards understanding disease pathophysiology in the context of the basal ganglia. However, as PD prevalence increases and lifespan increases as well, non-motor symptoms in PD are gaining increasing clinical and scientific relevance.

Non-motor symptoms in Parkinson's disease and dementia with Lewy bodies

While a major theme of his "Essay" was shaking palsy as a motor disorder, Parkinson also recognized and documented non-motor symptoms that remain relevant to this day. In particular, he noted sleep disturbances, constipation, dysphagia, and incontinence (Parkinson, 2002). Today, non-motor symptoms (NMS) are considered a critical aspect of

PD and may even present prior to the onset of motor symptoms, representing a prodromal stage of the disease (Postuma et al., 2012, 2015). Further support for non-motoric prodromal PD comes from postmortem studies of sporadic PD patients demonstrating that the pathologic process may begin in regions outside of the substantia nigra pars compacta (SNPc) such as the anterior olfactory nucleus and dorsal motor nucleus of the vagus nerve (Braak et al., 2003). In line with these pathological findings, hyposmia, impaired olfaction, is sensitive for PD (> 80%) but not specific (Postuma et al., 2012).

Braak staging demonstrating Lewy body pathology beginning in the medulla and pons is possibly associated with rapid eye movement (REM) sleep behavior disorder (RBD), a pathological condition where REM sleep is not accompanied by the typical atonia (lack of skeletal muscle activity) (Postuma et al., 2012). The clinical implications of sleep changes in the context of prodromal PD is highly significant as longitudinal studies demonstrate that RBD patients develop PD at rates that climb up to 90.9% at 25 years following diagnosis of RBD (St Louis and Boeve, 2017). Despite this high phenoconversion rate, it is important to note that RBD has been shown to occur in only 30 to 50% of PD patients (Schenck et al., 2013), suggesting that pathological processes mediating RBD may be contributing to, rather than essential for, PD pathogenesis.

Autonomic dysfunction is another NMS in PD and potentially also in the prodromal stage. Orthostatic hypotension and urinary incontinence represent two key products of autonomic dysfunction in PD, and these symptoms are found in both early and prodromal stages of the disease (Obeso et al., 2017; Postuma et al., 2012, 2013). A more prominent manifestation of autonomic dysfunction is gastrointestinal dysfunction, which occurs along the length of the gastrointestinal tract in PD, including excess salivation, dysphagia, and gastroparesis. Constipation and gastroparesis are common in individuals with PD (Postuma et al., 2013; Schapira et al., 2017) and are believed to be secondary to impaired activity of the enteric nervous system. Like RBD, support for gastrointestinal dysfunction

being a component of prodromal PD first comes from epidemiological studies documenting constipation representing an increased risk for PD (Savica et al., 2009), and is furthered by pathological studies demonstrating PD pathology in cells of the gastrointestinal tract and enteric nervous system (Braak et al., 2006; Chandra et al., 2017; Lebouvier et al., 2009). Moreover, the vagus nerve is important for autonomic control of the bowel, the dorsal motor nucleus of the vagus is one of the earliest sites of PD pathology (Braak et al., 2003), potentially explaining why gastrointestinal disturbances are a common non-motor feature of PD, but also may occur prior to alterations in motor function.

Cognitive decline: a prominent non-motor symptom in Parkinson's disease and other synucleinopathies

In James Parkinson's 1817 definition of the shaking palsy, cognitive impairment does not appear to be a prominent disease feature. This is highlighted in two of his six cases: "the senses and intellects being uninjured" in one, and another "the powers of his mind, unimpaired." Two centuries of clinical observations have advanced Parkinson's initial findings to now include cognitive decline and dementia as one of the most frequent non-motor problems in advanced stages of PD that greatly impairs quality of life (Winter et al., 2011). Together, PDD and DLB represent a multifactorial burden and challenge, increasing caregiver burden, health-related costs, risk for admission to a nursing home or assisted living facility, duration of hospital stays, and even mortality (Fletcher et al., 2011; Vossius et al., 2011).

Mild cognitive impairment (MCI) is often considered a precursor to dementia, where cognitive deficits are present and detectable via assessment, but, unlike dementia, are not severe enough to significantly impair daily life and one's ability to live independently. MCI is evident in approximately 10-20% of individuals at the same time they are diagnosed

with PD (Svenningsson et al., 2012), however not all individuals with MCI progress to dementia. PD patients have a three-to-six times higher risk of developing dementia than age-matched healthy individuals (Svenningsson et al., 2012). Cross-sectional studies have demonstrated that upwards of 30% of all patients with PD have dementia (Aarsland et al., 2008). The incidence of dementia in PD is 100 per 100,000 patient-years, with 80% of individuals with long-standing PD likely to develop dementia at some point during the course of the disease (Aarsland et al., 2017). Given that the mean age of PD diagnosis is near 60 years of age, and life expectancy for adequately-managed, non-demented PD individuals is similar to that of unaffected individuals (Winter et al., 2011), cognitive impairment and dementia in the α -synucleinopathies is a pressing clinical challenge.

Current treatments and the need for studying non-motor symptoms in Parkinson's disease and dementia with Lewy bodies

Loss of dopaminergic (DAergic) neurons in the SNPc leading to depletion of striatal dopamine (DA) loss underlies the cardinal motor features in PD. Since its identification in the 1960s, DA replacement in the form of levodopa (L-DOPA, the blood-brain-barrier-permeable precursor to DA) continues as the current gold standard in treatment of PD symptoms, attempting to mitigate decreased dopaminergic output from the SNPc. Current L-DOPA preparations include aromatic amino acid decarboxylase (AADC) inhibitors such as carbidopa in order to prevent metabolism of L-DOPA to DA in the periphery and enhance half-life and bioavailability in the brain. To complement AADC inhibitors, catechol-O-methyltransferase (COMT) inhibitors are also employed to further enhance DA half-life and brain bioavailability. In addition to reuptake from the synaptic cleft, DA is cleared through oxidation via monoamine oxidase B (MAO). As such, pharmacologic MAO inhibitors, such as selegiline are used to increase synaptic concentrations of DA. Beyond DA replacement and pharmacologic tools to support this process, DA agonists are also

palliative approaches to motor dysfunction in PD, acting on DA receptors of striatal medium spiny neurons. While DA agonists are marginally less effective as compared to L-DOPA, they are less prone to inducing striatal DA receptor stimulation as compared to L-DOPA, and thus less likely to induce unwanted motor side effects (dyskinesia). It is, however, important to note the DA replacement therapies are even incomplete in their capacity to treat motor dysfunction in PD, as many forms of tremor, freezing of gait, and postural instability are often resistant to L-DOPA therapy.

Deep brain stimulation (DBS) of the subthalamic nucleus or globus pallidus internus emerged in the 1990s for management of motor dysfunction in PD for patients whose symptoms were not adequately controlled pharmacologically (Okun, 2012). Importantly, DBS has proven to improve quality of life in PD patients by serving to reduce drug dosage needs and dyskinesia and improve activities of daily living. However, several non-motor symptoms found in PD are exclusion criteria for DBS, such as dementia, psychosis, and depression, and thus non-motor symptoms are not currently able to be managed via DBS.

Gene therapy also presents an area of clinical opportunity for therapeutic advance in PD. The two major strategies for gene therapy in PD is delivery of viral vectors to mediate expression of growth factors or neurotransmitter-synthesizing enzymes. Delivery of trophic factors such as glial cell line-derived neurotrophic factor (GDNF) and neurturin has proven beneficial in animal models of PD and certain clinical trials (Kordower and Bjorklund, 2013). Viral vector delivery of enzymes involved in DA synthesis such as TH or AADC have been injected into the striatum, with the goal of modifying striatal neurons to produce and release DA locally, compensating for SPNc neurodegeneration (Mittermeyer et al., 2012; Palfi et al., 2014). Fetal cell transplantation of DAergic neurons have also been shown to restore DA neurotransmission and connectivity in PD patients, but sadly no evidence of clinical benefit was reported (Freed et al., 2001). Active and passive

immunization focusing on α S have more recently come into the realm of clinical trials (Mandler et al., 2014).

While an improved knowledge of disease pathophysiology has led to considerable advances in PD therapies, there remains a focus on treating PD as a movement neurological disorder centered around the basal ganglia. The therapeutic limitation of DAergic therapies in PD comes from the Levodopa in Early Parkinson Disease (LEAP) trial, where L-DOPA had no disease-modifying effect (Verschuur et al., 2019). Moreover, all current DA-centric therapies fail to (Seppi et al., 2011) address NMS in PD, particularly cognitive decline. Treatments addressing dementia and cognitive dysfunction in PD and DLB are extremely limited primarily borrowed from AD via repurposing of anticholinesterase or and N-methyl-D-aspartate (NMDA) receptor inhibitors (Connolly and Fox, 2014; Seppi et al., 2011). While this approach has indeed provided some clinical benefit for dementia in the α -synucleinopathies, it is clear that meaningful therapeutic advances truly rely on a better understanding of the pathological mechanisms and processes underlying cognitive dysfunction in PDD and DLB.

Models for studying α -synucleinopathies

Clinical, pathological, and genetic studies have greatly informed our capacity to model α -synucleinopathies, PD in particular, both *in vitro* and *in vivo*. However, it is important to understand that disease models are likely to not be complete replications of the human disease and identifying the right model for the question being asked and understanding both the benefits and limitations of that model, are essential to drawing meaningful conclusions from experiments. Indeed, while an ideal model for PD would capture all aspects of the disease, none of the current models completely recapitulate the cardinal clinical and neuropathological findings observed in PD. This is not to say that any of these

current models are considered to be failures but are, instead, effective and versatile experimental tools used in pursuit of answering a scientific and clinical hypothesis. Despite these challenges in modeling PD and the α -synucleinopathies and not without caveats, these disease models have yielded significant insight into disease pathogenesis and served as platforms to test potential therapeutics and will continue to be major contributors in the quest towards better understanding disease mechanisms and developing treatment interventions.

Some of the initial animal models used to investigate PD were born out of a connection between toxins and parkinsonism. The identification of MPTP-induced parkinsonism, specific DAergic neuron loss, and Lewy pathology in patients (Langston et al., 1983) established MPTP as a tool to study the mechanisms underlying DAergic neuron death in disease. First generation MPTP animal models consisted of intravenous MPTP injections into primates. These monkeys developed almost all of the motor symptoms observed in PD patients, were responsive to levodopa, and also had DAergic neuron loss (Burns et al., 1984). Mice injected with MPTP also demonstrated destruction of the nigro-striatal pathway, loss of DAergic neurons in the SNPc, and locomotor abnormalities (Jackson-Lewis et al., 1995; Muthane et al., 1994; Taylor et al., 2010). However, Lewy pathology (LBs and LNs) are noticeably absent in the MPTP toxin model (Halliday et al., 2009). Despite this shortcoming, MPTP-induced neurodegenerative changes are specific and reproducible, a major strength when considering the landscape of genetic models of PD and the α -synucleinopathies. Additional toxin models were informed by epidemiological studies connecting the pesticides rotenone and paraquat with early-onset parkinsonism (Tanner et al., 2011). Chronic exposure to rotenone in rats has proven to induce degeneration of DAergic nigro-striatal neurons and postural and motor abnormalities resembling the parkinsonian phenotype (Betarbet et al., 2000). Rotenone-

injected rats also display α -synuclein- and ubiquitin-positive cytoplasmic inclusions in nigral neurons reminiscent of Lewy bodies (Betarbet et al., 2000; Sherer et al., 2003). The rotenone model is exciting for its ability to reproduce these key neuropathological and locomotor findings, but faces challenges with variability between animals and high mortality (Betarbet et al., 2000). Systemic application of the herbicide paraquat has been shown to impair locomotor activity due to DAergic neuron and striatal tyrosine hydroxylase (TH) loss (Brooks et al., 1999). 6-OHDA functions similarly to MPTP as a neurotoxin selective for dopaminergic neurons. However, in contrast to MPTP and other neurotoxins, 6-OHDA is blood-brain-barrier impermeable and thus requires direct injection into the striatum to exert its pro-parkinson effects that include DAergic neuron loss in the SNPc (Sauer and Oertel, 1994). Collectively, these models, directly informed by clinical and epidemiological observations, represent a platform to provide insight into the mechanisms driving selective neuron loss in PD, traditionally through a focus on differential bioenergetic demands and mitochondrial dysfunction (Halliday et al., 2009; Meredith and Rademacher, 2011; Surmeier et al., 2010). While potentially limited in disease scope, the toxin animal models are the only systems currently employed that provide for the development SNPc DAergic neuron loss.

The identification of genetic mutations in the late 1990s as a cause for inherited forms of PD provided for the generation of transgenic models. There are two known autosomal-dominant inherited causes of PD: mutations in the *SNCA* gene encoding α S and mutations in the *LRRK2* gene encoding the leucine rich repeat kinase 2 (LRRK2) protein. *SNCA* gene duplications, triplications, or point mutations (A30P, E46K, H50Q, G51D, and A53T) cause familial PD, and many of these genetic abnormalities have been used to direct transgenic animal model generation. However, while *SNCA* was the first gene linked to familial autosomal-dominant PD (Polymeropoulos et al., 1997), its product, α S, is the main

component of LBs and LNs, found in both familial and idiopathic cases of PD (Spillantini et al., 1997). As such, while using α S-based transgenic mouse models for PD may be based on rare mutations, they have the potential to illuminate mechanisms of α S-mediated neuronal dysfunction and neurodegeneration in all cases of PD and DLB.

Some of the first genetic-derived *in vivo* models of PD were generated in the fruit fly (*Drosophila melanogaster*). Although *Drosophila* do not have an *SNCA* homolog, they present a unique and versatile model for studying PD pathogenesis given their rapid generation cycle (10-14 days from embryo to adult), short lifespan (50-60 days), relatively high homology to human genes (75%), and expression of dopaminergic neurons (Mizuno et al., 2010). Overexpression of h α S^{WT}, h α S^{A30P}, or h α S^{A53T} in *Drosophila* causes TH loss, neuronal cytoplasmic filamentous inclusions composed of α S, retinal neurodegeneration, and motor deficits (Feany and Bender, 2000). Interestingly, the parallel time course of dopaminergic neuron loss, α S inclusion formation, and behavioral deficits suggests that these three abnormalities and hallmarks of PD, are linked. *Drosophila* has proven to be a fruitful model for insight into the role of α S in PD, connecting α S with TH neuron loss, and phosphorylation of α S at serine 129 increasing toxicity and inclusion formation (Auluck et al., 2010; Chen and Feany, 2005). Another non-rodent animal model of PD that has helped to advance our understanding of PD has been the nematode (*Caenorhabditis elegans*). Early *C. elegans* model systems used relied on neuronal-specific promoters to direct either human WT or mutant α S overexpression and have been valuable for demonstrating α S-mediated dopaminergic neuron loss as a key driver of locomotor dysfunction (Lakso et al., 2003).

Advancing in terms of complexity, mouse models of disease have become increasingly common given the extremely high homology (~98%) to the human genome (Guénet, 2005). To date, many α S transgenic (Tg) mouse models (overexpressing human WT or

mutant α S) via different promoters have been developed and remain a staple of PD research. While these mice may display decreased levels of TH or DA in the striatum, behavioral abnormalities, and cytoplasmic α S aggregates, degeneration of nigro-striatal DAergic neurons has not been observed. The first Tg mouse model of PD expressed $h\alpha S^{WT}$ under the PDGF- β promoter (PDGF- $h\alpha S^{WT}$), targeting transgene expression to neurons (Masliah et al., 2000). While these mice display ubiquitin and α S-positive intraneuronal inclusions, TH fiber and DA reduction in the striatum, and impaired motor performance, neurodegeneration is markedly absent. Tg mice expressing $h\alpha S^{WT}$ under the mouse Thy-1 promoter (Thy-1- $h\alpha S^{WT}$) demonstrated a higher and more broad expression of human α S throughout the nervous system, including basal ganglia and SN when compared to PDGF- β transgenic mice (Rockenstein et al., 2002). Thy-1- $h\alpha S^{WT}$ Tg mice also displayed intracellular α S inclusions in the SN, and low expression of α S in glia, consistent with α S being expressed almost exclusively in neurons, in contrast to what was observed in the PDGF- $h\alpha S^{WT}$ Tg model. In addition to promoter differences, it is also important to take the genetic background of the mouse strain used into consideration. The Tg mice previously discussed were both on the C57BL6/J x DBA mouse strain background. In contrast, an additional set of Thy-1- $h\alpha S^{WT}$ and Thy-1- $h\alpha S^{A30P}$ Tg mice under on the C57BL6/J mouse background alone display lower SNpc α S pathology but higher pathology and neurodegeneration in motor neurons (van der Putten et al., 2000). Generation of Thy-1- $h\alpha S^{A30P}$ Tg mice on the C57BL/6 background display several cardinal PD features including fibrillar LB-like inclusions, reduction in DA levels, and late-onset paralysis (Kahle et al., 2000; Neumann et al., 2002). However, neurodegeneration in these Thy-1- $h\alpha S^{A30P}$ Tg mice is found outside of the basal ganglia, localized to sensory and motor neurons in the brainstem and spinal cord (Neumann et al., 2002), While this

collection of murine Thy-1 Tg mice lack overt loss of nigro-striatal DAergic neurons, many show loss of DA reduction (van der Putten et al., 2000; Rockenstein et al., 2002).

Like Thy-1, the mouse prion promoter (mPrP) has also been a useful tool in driving pan-neuronal transgene expression. Homozygous Tg mice expressing human missense mutant A53T α S under the mPrP promoter (mPrP-h α S^{A53T}, *line M83*) display progressive motor impairment and diffuse LB-like α S pathology throughout the mouse brain (Giasson et al., 2002). Another mPrP-h α S^{A53T} Tg mouse replicates the pan-neuronal expression of human α S and deficits in motor coordination and performance, but no aggregation (Gispert et al., 2003). It is, however, important to note that these mice were on a different, FVB/NJ, mouse background. A caveat to the FVB/NJ model is retinal degeneration leading to blindness (Wong et al., 2012).

An exciting advance in the era of α S Tg mouse models in PD research came from the generation and comparison of mPrP-h α S^{WT} (TgWT, *line I2-2*), mPrP-h α S^{A30P} (TgA30P, *line O2*), and mPrP-h α S^{A53T} (TgA53T, *line G2-3*) in the C57BL6/J background (Lee et al., 2002). The generation of these Tg mice all on similar backgrounds provided for a direct comparison of α S variants and expression. In addition to the G2-3 line, a lower expressing h α S^{A53T} line was generated, *line H5*. Both G2-3 and H5 mice develop progressive neurological abnormalities and premature death, but higher transgene expression was associated with earlier disease onset and death (Lee et al., 2002), in line with the observation that α S expression is a key determinant of PD progression (Singleton et al., 2003). O2 mice, while expressing similar levels of transgene when compared to G2-3, do not develop overt neuropathology or neurological abnormalities. G2-3 mice also display ubiquitin- and α S-positive intracellular inclusions in deep cerebellar nuclei, reticulo-pontine nuclei, spinal cord, motor neurons, and neocortex, but not the SNPc. In line with lack of SNPc pathology, there is neither loss of DAergic neurons or DA in the striatum. Similar to

the O2 strain, I2-2 mice do not develop neurological dysfunction, suggesting that the missense A53T mutation in human α S is associated with increased neurotoxicity in mice.

Human α S (WT, A30P, or A53T) transgene expression has also been focused to the striatum via use of the TH promoter. Similar to Tg mice with pan-neuronal Thy-1 or mPrP promoters, TH Tg mice do not entirely capture the human version of PD and accompanying α -synucleinopathies. Initial studies from these mice demonstrate that despite high α S transgene expression, there is a lack of neuropathological or behavioral changes as indicated by an absence of Lewy-like inclusions, reduced striatal DA, or loss of nigral neurons (Matsuoka et al., 2001). While other iterations of the TH Tg mice displayed some modest neurological changes, including dystrophic neurites, reduced DA, and impaired motor coordination, intraneuronal Lewy-like aggregates of α S were noticeably absent (Richfield et al., 2002).

Improved genetic tools have provided for newer models of PD and the α -synucleinopathies. An example of this is the tetracycline (tet)-regulated transgenic switch that employs two separate components acting in *trans*: a tissue-specific promoter (activator or driver component transcribing the tet-regulated transcription activator, tTA) plus responder component (transgene of interest under the tTA promoter) (Gossen and Bujard, 1992). tTA affinity for the target promoter can be altered, activated or inactivated, via tetracycline antibiotics such as doxycycline, providing for temporal control of transgene expression in addition to regional control. Tg mice overexpressing h α S^{WT} or h α S^{A53T} in the tet-OFF CaMKII conditional promoter system have targeted transgene expression to forebrain and midbrain neurons (Nuber et al., 2008). Neurodegeneration in these strains is observed in the SNpc, hippocampus, and non-DAergic neuron loss, modest loss of TH-positive cells, but no α S Lewy-like pathology. These mice also display impaired motor learning and function as assayed via rotarod, but normal DA levels in the striatum. Another

tet-OFF system Tg-h α S^{A53T} mice under the Pitx3 promoter targets transgene expression to midbrain DA neurons (Lin et al., 2012). Pitx3-directed expression of h α S^{A53T} results in pronounced midbrain neurodegeneration, reduction in DAergic neurotransmission, and motor dysfunction. With these deficits and regionally-focused expression, use of the tTA system, in contrast to pan-neuronal promoters of the Thy-1 and mPrP mice, provides a powerful tool to parse out the brain region specificity and contributions to the neuropathological, biochemical, functional, and behavioral abnormalities occurring in these mice and potentially in PD brains as well.

While at first blush the lack of a complete recapitulation of all aspects of PD in these Tg mice may be interpreted to be a shortcoming in disease modeling, they provide an incredibly valuable window into the disease. These animals represent essential tools in advancing our understanding of key pathological processes and mechanisms driving the molecular, cellular, and behavioral changes underlying neurodegenerative disorders, and the α -synucleinopathies in particular.

Cognitive decline in mouse models of Parkinson's disease and dementia with Lewy bodies

NMS in PD, including psychiatric and sleep disorders, and cognitive, gastrointestinal, and autonomic dysfunction, are garnering increasing attention as they continue to remain unresolved by DA-centric therapies such as L-DOPA and DBS (Chaudhuri and Schapira, 2009). As these treatments have been able to largely and successfully provide symptomatic relief from these cardinal motor symptoms, NMS often have a greater negative impact on quality of life when compared to motor dysfunction (Martinez-Martin et al., 2011). Motivated by the adverse influence of NMS in the quality of life in PD patients, it has been of interest to assess whether animal models of PD can be used to investigate the mechanisms driving NMS in PD and the α -synucleinopathies.

Toxin models of parkinsonism are popular models for their capacity to produce DAergic neuron degeneration in the nigro-striatal pathway and motor dysfunction (Meredith and Rademacher, 2011). Bilateral injection of 6-OHDA into the SNPc of rats led to striatal loss of DA neurons, reduction in TH immunoreactivity, and the development of spatial memory deficits (Tadaiesky et al., 2008). In addition to producing cognitive dysfunction, this toxin model and approach produces partial replication of psychiatric NMS, including a depressive-like phenotype, that is likely due to reductions in DA, serotonin (5-HT) and noradrenaline (NA), demonstrating that 6-OHDA can induce changes in other neurotransmitter systems beyond DA (Santiago et al., 2010). However, whether these changes are a direct consequence of 6-OHDA neurotoxicity or due to neurodegenerative changes in DAergic circuitry remain to be elucidated. Systemic MPTP injection presents an established model for nigro-striatal DAergic neurodegeneration and parkinsonism in mice. MPTP-treated mice have been shown to develop deficits in cued fear conditioning that is accompanied by decreased DA and 5-HT levels in the striatum, amygdala, and cortex (Vučković et al., 2008). While these 6-OHDA and MPTP models do replicate several non-motor aspects of PD, particularly cognitive impairment, the rapid onset of neurodegeneration does not accurately reflect the more global and progressive nature of the disease.

Patients with familial PD due to *SNCA* duplications, triplications, or missense mutations including A30P, E46K, and A53T often have prominent NMS, including cognitive impairment and dementia (Bougea et al., 2017; Ikeuchi et al., 2008; Kruger et al., 2001; Puschmann et al., 2009; Singleton et al., 2003; Spira et al., 2001; Zarranz et al., 2004). A53T α S carriers have also presented with cognitive decline resembling FTD prior to overt parkinsonism onset (Bougea et al., 2017). In addition to causal mutations in *SNCA*, different *SNCA* haplotypes associated with an increased risk for PD are also associated with impaired cognitive performance and learning (Kéri et al., 2008). Taken

together, these observations provide compelling evidence for α S-mediated cognitive decline in PD and DLB, a hypothesis that can be directly tested in α S genetic models of PD.

PDGF- $h\alpha$ S^{WT} Tg mice display a progressive loss of DAergic neuron terminals in the striatum and is accompanied by locomotor abnormalities (Masliah et al., 2000). Cognitive function, as assayed via the Morris water maze, is impaired by six months of age and given that these mice display extensive cortical, hippocampal, and limbic α S pathology, the PDGF- $h\alpha$ S^{WT} mouse is preferentially characterized as a DLB model rather than PD (Rockenstein et al., 2002). Thy-1- $h\alpha$ S^{WT} Tg mice display impaired performance in the Y-maze spontaneous alternation and novel object recognition tasks beginning as early as four months of age, prior to neurodegenerative changes in the striatum and SNPC (Chesselet et al., 2012; Magen et al., 2012). More recently, 5-month-old Thy-1- $h\alpha$ S^{WT} mice have been shown to display cognitive deficits in the short-term spatial learning and memory setup of the Y maze (Ferreira et al., 2017). It is interesting to note that while these mice all express $h\alpha$ S^{WT}, their genetic backgrounds, promoter, transgene expression level, and α S-induced neuropathology and neurodegenerative phenotypes differ slightly. However, as they all have been reported to display deficits in cognitive performance, albeit in different experimental paradigms and at different age points, one can hypothesize that neuronal and synaptic mechanisms underlying memory impairment in these mice is independent of overt PD-associated neuropathology.

Many strains of Tg mice expressing human mutant α S have been shown to display increased neuropathology and neurotoxicity as compared to their $h\alpha$ S^{WT} counterparts, and thus it could be reasonably hypothesized that these strains demonstrate more robust NMS. Thy-1- $h\alpha$ S^{A30P} Tg mice display progressive, age-dependent cognitive deficits on the Morris water maze and via cued fear conditioning by 12 months of age, that appear to

coincide with development of fibrillar intraneuronal cytoplasmic α S inclusions in both the hippocampus and amygdala (Freichel et al., 2007). $h\alpha S^{A30P}$ -mediated deficits in contextual fear conditioning have been observed in much older mice, averaging 18-months-old, and were accompanied by increased pSer129- α S staining (Schell et al., 2012). 12-month-old mPrP- $h\alpha S^{A53T}$ (M83) mice display age-dependent spatial memory deficits when tested in the Y-maze, but not at younger age points (Paumier et al., 2013). Both mPrP- $h\alpha S^{WT}$ (I2-2) and mPrP- $h\alpha S^{A53T}$ (G2-3) display cognitive deficits in spatial learning and memory as assessed via Barnes maze (Larson et al., 2012; Teravskis et al., 2018), yet I2-2 mice do not display neuropathology or neurotoxicity as compared to the G2-3 (Lee et al., 2002), contributing another layer to the argument that cognitive dysfunction in these mice and in PD may not be mediated by Lewy-like aggregates or DAergic neurodegeneration. Instead, soluble oligomeric species of α S have been demonstrated to impair synaptic structure, synaptic plasticity, and learning and memory in mice (Castillo-Carranza et al., 2018; Ferreira et al., 2017; Larson et al., 2012, 2017; Martin et al., 2012; Sengupta et al., 2015)

In contrast to the Tg mice above all using promoters that drive pan-neuronal expression, use of the CaMKII promoter restricts expression to forebrain structures. This represents an ideal system for assessing the consequences of regional $h\alpha$ S expression, such as learning and memory deficits in the hippocampus due to specific $h\alpha$ S expression in cortical and hippocampal neurons. Tg CaMKII-tTA mice expressing $h\alpha S^{WT}$ (CaMKII-tTA- $h\alpha S^{WT}$) display impaired memory function on the Morris water maze at 12 months of age and hippocampal α S pathology at 20 months (Nuber et al., 2008). CaMKII-tTA- $h\alpha S^{A53T}$ mice also display age-dependent cognitive deficits as measured via contextual fear conditioning that is partly rescued by $h\alpha S^{A53T}$ suppression (Lim et al., 2011). Given that forebrain-directed $h\alpha$ S expression is sufficient to induce progressive cognitive deficits, it is possible to conclude that DAergic neurodegeneration is not a major driver of α S-

mediated memory dysfunction but rather due to α S-induced abnormalities in cortical and hippocampal neurons and their synapses. This is supported by studies demonstrating that overexpression of $h\alpha S^{WT}$ contributes to memory deficits in transgenic mouse models that lacking midbrain neurodegeneration (Larson et al., 2012, 2017; Magen et al., 2012).

Cell and region-specific overexpression of $h\alpha S$ in midbrain DAergic neurons is achieved through use of the TH promoter or the Pitx3-tTA system. While DA and motor abnormalities have been observed in TH- $h\alpha S^{WT}$ Tg mice, cognitive deficits, to date, have not been reported (Richfield et al., 2002). In a similar vein, Pitx3-tTA- $h\alpha S^{A53T}$ animals display gait and motor deficits as early as two months of age that is accompanied by neuronal and DA loss neuron and striatal DA loss by six months (Lin et al., 2012). However, it is not known if these mice display any cognitive impairments, potentially suggesting that alterations in other circuits and neurotransmitter systems may be the predominant factors in driving cognitive decline in PD and DLB.

The causal relationship between the A53T missense mutation in the human *SNCA* gene and PD lends itself to the question if mice, which naturally have threonine (T) at residue 53 in the α S protein, are predestined to develop a rodent form of parkinsonism. In mice overexpressing mouse wild-type α S ($m\alpha S^{WT}$) under the control of the Thy-1 promoter (Thy-1- $m\alpha S^{WT}$) (Rieker et al., 2011), transgene expression was observed throughout the nervous system, similar to Thy-1- $h\alpha S^{WT}$ animals (Chesselet et al., 2012). Moreover, α S pathology, as demonstrated by ubiquitin and pSer129 staining was observed in both brain stem and spinal cord regions of mice, similar to G2-3 and M83 animals (Giasson et al., 2002; Lee et al., 2002). Interestingly, pSer129 also accumulated in hippocampal neurons of Thy-1- $m\alpha S^{WT}$ mice. However, in contrast to the range of symptoms and phenotype displayed by mice expressing $h\alpha S^{A53T}$, overexpression $m\alpha S^{WT}$ does not induce early-onset behavioral changes by comparison, such as cognitive decline, but does drive similar

end-stage pathophysiological deficits such as mild synaptic and locomotor dysfunction (Rieker et al., 2011). Taken together, this study suggests that cognitive deficits in mouse models of PD is more likely a product of specific h α S expression associated with PD as opposed to general overexpression of the protein.

Transgenic mouse models provide a vital role in the investigation of α -synucleinopathy pathophysiology but rely on a non-physiologic promoter of only complementary DNA (cDNA) that, together, may preclude the capacity to control any transcriptional or post-transcriptional modifications involved in disease. As such, the generation of PD mouse models using bacterial artificial chromosomes (BAC) using the full gene sequence and endogenous promoter provide a more native spatial and temporal expression pattern and ideally create more physiologic-relevant system. While some of these models display modest DAergic neuropathological and neurodegenerative phenotypes observed in PD and other Tg models, NMS, including cognitive impairment, is largely absent (Janezic et al., 2013; Kuo et al., 2010). The modest phenotype observed in BAC lines is likely due to their low expression levels of h α S.

The TgA53T (G2-3) mouse model of α -synucleinopathy

G2-3 mice (Lee et al., 2002) represent one of many Tg mouse models expressing h α S^{A53T}, but has become a staple strain in the investigation of PD pathophysiology. Broadly speaking, it appears that h α S^{A53T} appears more effective, or toxic, in mice as compared to overexpression of h α S^{WT} or h α S^{A30P}. Pan-neuronal transgene expression, driven by the mPrP promoter, produces α S at levels about six-times higher than endogenous m α S. However, unlike other TgA53T α S mice such as M83 (Giasson et al., 2002), homozygous G2-3 mice are not viable, and thus all G2-3 mice studied are hemizygous for their transgene.

The initial reporting and characterization of G2-3 mice demonstrated that these mice develop progressive motor impairments around 10 months of age leading to premature death by 12 months of age (Lee et al., 2002). Early signs of motor impairments include wobbling and hindlimb retraction when picked up by the tail. This is followed by decreased locomotion, and mice ultimately become no longer able to walk upright and move, followed by hindlimb paralysis and cachexia en route to disease “end-stage” (“sick”). However, this time frame has since been shifting as this motor phenotype is about 90% penetrant, with “escaping” mice surviving to around 18 months (Jackson Laboratory, stock #006823, January 2019).

Use of the mPrP promoter drives $h\alpha S^{A53T}$ expression throughout the nervous system, including the SNPc. While *in situ* hybridization also confirmed mRNA in glia, levels were significantly reduced as compared to neurons (Lee et al., 2002). Consistent with αS being a presynaptic protein, $h\alpha S^{A53T}$ was detected in a punctate pattern in neurons and the neuropil, suggesting that trafficking and localization of the protein in mouse neurons remains physiological despite marked overexpression. Tg expression is also found in the SNPc, but neuropathology and neurodegeneration are noticeably absent from this region. Older and end-stage mice demonstrate accumulation of αS in other neuronal populations, however, including the midbrain, cerebellum, brainstem, and spinal cord motor neurons. In addition to being positive for αS , these aggregates are also immunoreactive for ubiquitin and neurofilament, and thioflavin-S-positive, indicative of a fibrillar structure similar to Lewy bodies (Lee et al., 2002). Reactive astrocytosis, and gliosis in general, is another feature in these mice, and is observed in regions with αS pathology such as the midbrain, brainstem, and spinal cord, but absent in regions without pathology such as the hippocampus and striatum.

In addition to late-stage motor impairments and premature lethality, G2-3 mice display progressive locomotor hyperactivity between six and nine months (Daher et al., 2012; Smith et al., 2010; Unger et al., 2006). Unger and colleagues went on to demonstrate that this hyperactivity phenotype was shown to be a product of increase DA D1 receptor expression in the striatum and reduced DA transporter (DAT) at the synapse, which was accompanied by a reduced rate of DA uptake from the synaptic cleft. Consistent with the absence of significant neuron loss in the striatum, levels of DA and its metabolites DOPAC and HVA remain unchanged as compared to non-transgenic (nTg) littermate controls (Lee et al., 2002). This latter point has been recently under review as one study has demonstrated h α S^{A53T}-mediated impairment of autophagy as measured by reduced LC3-II signal in the striatum and SNPC, which is accompanied by TH reduction in the striatum (Pupyshev et al., 2018). However, Pupyshev et al. (2018) also found a reduction in striatal DAT expression, similar to Unger et al. (2006). Although locomotor hyperactivity may not be a direct clinical observation in PD patients or those with parkinsonism, the G2-3 model and these studies clearly demonstrate that h α S^{A53T} expression is capable of disrupting DA metabolism and neurotransmission, and DAergic neurons as a whole, in line with what is observed in PD. Accompanying progressive these DAergic locomotor abnormalities, cognitive dysfunction at older ages like 11-12 months (Teravskis et al., 2018)

G2-3 mice have provided valuable insight into the mechanisms of α S-induced neuronal dysfunction. h α S^{A53T} demonstrates increased protein half-life, or decreased degradation rate, as compared to h α S^{WT} h α S^{A30P} variants (Li et al., 2004). It is therefore possible that this differential metabolism of h α S^{A53T} and age-dependent pathogenicity in when expressed in mice (Lee et al., 2002), and that toxicity is not just a product of increased translation of the protein, but stabilization of this translated product contributes to increased protein levels. G2-3 mice have been utilized to strengthen the mechanisms

connecting aging to neurodegenerative processes and have also been able to expand upon *in vitro* observations of h α S^{A53T}. In addition to α S aggregates, both G2-3 and M83 mice display α S species at lower molecular weights than full-length around 14 kDa (Giasson et al., 2002; Lee et al., 2002). Moreover, these lower molecular weight species have been reported to be truncated on their C-terminus, a post-translational modification that enhances fibril assembly *in vitro* (Murray et al., 2003; Serpell et al., 2000). These C-terminal truncated α S species are aggregation-promoting, pointing to another mechanism for α S-mediated abnormalities in neuronal dysfunction and PD pathogenesis (Li et al., 2005).

Further insights into the mechanisms underlying α S-mediated neurodegeneration have been directly informed by studies in G2-3 animals. In line with proteostatic abnormalities being a core tenant of h α S^{A53T}-pathogenicity, G2-3 neurons accumulate toxic α S oligomers and are prominent in end-stage animals (Colla et al., 2012a). In particular, these oligomers preferentially localize to the endoplasmic reticulum (ER) and contribute to pathological ER stress, further impairing the cellular protein-folding machinery and rendering neurons more susceptible to accumulation of additional misfolded proteins. Along these lines, the unfolded protein response, while activated in response to α S aggregates, is dysfunctional in G2-3 mice and may also contribute to increased h α S^{A53T} accumulation and aggregation as animals age (Colla et al., 2012b). While ER chaperones are activated in G2-3 animals in a likely attempt to mitigate α S misfolding and aggregation, this ultimately leads to α S accumulation in the ER, ubiquitination, caspase activation, and ER stress-induced neurotoxicity, highlighting a pathway by which h α S^{A53T} contributes to neuronal dysfunction *in vivo*. Another component to protein folding are heat shock proteins (HSP), molecular chaperones that serve to ensure correct folding of proteins. Interestingly, G2-3 mice display increased levels of

HSP25 in reactive astrocytes found in regions of α S pathology such as the brainstem and spinal cord (Wang et al., 2008). By comparison, HSP25 levels were less prominent in neurons in these affected regions, suggesting that glial upregulation of HSP25 may represent a protective mechanism against neuronal dysfunction and degeneration in PD.

Additional pathological post-translational modifications of α S and the mechanisms driving them have also been investigated *in vivo* through the use of G2-3 animals. Activation of the tyrosine kinase c-Abl has been observed in the striatum and SNpc in human PD brains, and more recently G2-3 mice, where c-Abl hyperactivation leads to increased phosphorylation of h α S^{A53T} on tyrosine 39, with neurotoxic consequences including increased α S aggregation, brainstem neuropathology, and mortality (Brahmachari et al., 2016). In addition to pathological gain-of-function modifications of h α S^{A53T} that contribute to its aggregation, h α S^{A53T} contributes to disease pathogenesis by altering physiological protein interaction and function. For example, G2-3 mice display reduced LC3-II expression, indicative of impaired autophagic function (Pupyshev et al., 2018). Earlier studies also shed light onto defective autophagy in this model. Synphilin-1 has been shown to promote α S clearance via autophagy but appears to be defective and co-aggregate with α S in Lewy bodies in PD brains (Wakabayashi et al., 2000). G2-3 mice crossed with synphilin-1 overexpressing Tg mice display attenuated locomotor hyperactivity and delayed mortality, suggesting that h α S^{A53T} partly exerts its neurodegenerative effects via impairment of synphilin-1 function (Smith et al., 2010). In addition to behavioral rescue, these bigenic mice display increased markers of autophagy (beclin-1 and LC3-II) and decreased pro-apoptotic markers (caspase-3 activation), further suggesting that the A53T mutation leads to the disruption of protein-protein interactions essential to normal proteostatic processes like autophagy (Smith et al., 2010).

Complementing these previous studies investigating the ER, autophagy, and proteasome, the potential role for mitochondrial dysfunction in PD pathogenesis has also been investigated through the use of G2-3 animals. Mitochondrial dysfunction has previously been linked to PD through several key observations: 1) MPTP inhibits complex 1 of the electron transport chain on mitochondria; 2) mutations in proteins involved in mitochondrial dynamics, PINK and parkin, are linked to familial autosomal recessive forms of PD. G2-3 mice have been employed to define a mechanistic connection between mutant α S, mitochondrial dysfunction, and neurotoxicity in PD, with a recent study taking a comparative approach between Tg $h\alpha S^{A53T}$ *Drosophila* and G2-3 mice models of α -synucleinopathy. $h\alpha S^{A53T}$ is shown to destabilize spectrin *in vivo*, a key protein involved in plasma membrane and cytoskeleton maintenance, impairing actin dynamics that ultimately disrupt proteins involved in key mitochondrial processes, specifically Drp1 and mitochondrial fission (Ordonez et al., 2018).

The Tg mouse has also provided a system to test the relationship between key proteins involved in both familial and sporadic forms of PD. *LRRK2* mutations (missense G2019S) also cause familial autosomal dominant PD with neurochemical and neuropathological findings that are essentially identical to that of familial of PD due to α S mutations or idiopathic PD, especially α S-positive LBs and LNs. As such, crossing G2-3 mice with G2019S-LRRK2 or LRRK2-knockout provided for the direct investigation of the role LRRK2 plays in α S-mediated neuro changes in mice. Surprisingly, neither overexpression of PD-associated mutant LRRK2 nor knocking out of endogenous mouse LRRK2 in G2-3 mice exacerbates or rescues the $h\alpha S^{A53T}$ -mediated disease phenotype, respectively (Daher et al., 2012), suggesting that the neurodegenerative phenotype observed in G2-3 is largely LRRK2-independent. It is therefore possible that LRRK2 may be upstream of α S in this specific disease cascade, and that $h\alpha S^{A53T}$ overexpression

masks the effects mutant G2019S-LRRK2 overexpression within the same neuron and animal. However, mice co-expressing of G2019S-LRRK2 and h α S^{A53T} under the forebrain-specific CaMKII promoter display enhanced neuropathology, but even in this mice it appears that the neuronal and pathological changes observed are largely dependent on α S (Lin et al., 2009).

Mutations in PINK or parkin are responsible for familial autosomal recessive (juvenile) forms of PD. While PD patients with these mutations display the majority of PD-defining features, cases of PD due to parkin mutations characteristically lack Lewy pathology (Dawson and Dawson, 2010). LBs are immunoreactive for both α S and ubiquitin, and α S has been shown to be ubiquitinated by parkin, an E3 ubiquitin ligase, leading to the hypothesis that functional parkin promotes LB formation (Shimura et al., 2001). Parkin has also been shown to localize to LBs in PD (Schlossmacher et al., 2002). The autosomal recessive inheritance pattern of PINK and parkin mutations in PD is suggestive for a loss of function and thus parkin-deficient mice have been used as animal models of PD (Goldberg et al., 2003) and G2-3 mice have been utilized to examine the contributions of parkin to α S-mediated neurodegeneration. Homozygous knockout of parkin in G2-3 mice did not reveal any appreciable biochemical, pathological, or behavioral differences as compared to G2-3 controls, suggesting that h α S^{A53T}-mediated neurodegenerative features in mice do not require parkin (von Coelln et al., 2006). As such, it is reasonable to conclude that, while mutant α S-induced PD and parkin-induced PD produce common final outputs of parkinsonism and SNPc neurodegeneration, they achieve this via differing mechanisms.

One caveat to the transgenesis approach used in generating many of the mouse models for PD including the G2-3 and M83 strains is the random insertion in the transgene within the mouse genome following pronuclear injection into single cell mouse embryos.

This randomness may partly account for differential expression levels of identical promoter-transgene combinations like that of the G2-3, H5, and N2.5 lines all expressing h α S^{A53T} under mPrP (Lee et al., 2002). One extreme example relevant to Tg models of neurodegenerative diseases is the rTg4510 mouse model of fronto-temporal dementia (FTD) and AD, that expresses human missense mutant P301L tau (SantaCruz et al., 2005). However, insertion of the P301L tau transgene produces a 244 kb deletion that disrupts the *Fgf14* gene and production of fibroblast growth factor 14 (FGF14), a protein involved in modulation of neurotransmission and also implicated in neurological disorders including ataxias and schizophrenia, and as a result the findings in the Tg4510 mouse cannot be exclusively linked to mutant tau as previously thought (Goodwin et al., 2019). The h α S^{A53T} transgene insertion site in G2-3 mice has been located to chromosome 10. However, this insertion leads to a 48 kb deletion that disrupts *2310039L15Rik*, a gene that encodes for a long intervening non-coding RNA (lincRNA) (Goodwin et al., 2019). While the function of this specific lincRNA is currently unknown, ncRNA are generally classified as functional RNA molecules that do not code for translation into a protein (“non-coding”), and do not overlap with protein-coding genes, but serve as regulators of gene transcription and post-transcriptional regulation (Ulitsky and Bartel, 2013). The consequence of this deletion in G2-3 mice, however, awaits further study.

Parkinson’s disease pathogenesis, α S propagation, and new avenues for disease modeling

Lewy pathology is distributed throughout the PD brain but preferentially localizes to the SNPc, amygdala, frontal cortex, parietal cortex, and temporal cortex (Halliday et al., 2011). It is therefore likely that the degenerative process advances throughout the nervous system along specific anatomic pathways (Braak et al., 2003). This Braak staging scheme for PD pathology posits that inclusions are likely found in the olfactory bulb and dorsal

motor nucleus of the vagus nerve, and then follow an ascending pattern through brainstem to reach midbrain and finally the cortex, gradually increasing in more brain regions as the disease progresses (Braak et al., 2003). Instead of each individual neuron within these regions developing α S pathology near-simultaneously, these pathological observations have supported the concept for pathologic α S aggregates being a transmissible species between neurons that follow defined, connected neuroanatomical pathways (Angot et al., 2010). Several observations support the idea of α S propagation and PD pathology spreading in a prion-like mechanisms. Fetal DA cell grafts transplanted into the striatum of PD patients have been shown to develop PD pathology (Kordower et al., 2008; Li et al., 2008). The pathological hallmark of another α -synucleinopathy, MSA, are GCIs, yet oligodendrocytes do not produce α S under normal or pathological states (Miller et al., 2005), adding to the concept of α S propagation between neurons and now also other nervous system cells such as glia.

Motivated by pathological studies demonstrating that α S aggregates in LBs and LNs adopts a β -amyloid-rich fibrillar confirmation (Spillantini et al., 1998b), the *in vitro* production of α S pre-formed fibrils (PFFs) using human WT α S has emerged as a tool for studying α -synucleinopathy processes both *in vitro* and *in vivo* (Volpicelli-Daley et al., 2014). Initial studies demonstrated that PFFs could induced pathology in transgenic mice overexpressing h α S (Desplats et al., 2009; Luk et al., 2009). Follow-up studies demonstrated that α S PFFs are capable of entering neurons via endocytosis and serving as a template for the induction pathology in cells expressing endogenous levels of α S by recruiting endogenous α S into newly-forming aggregates (Volpicelli-Daley et al., 2011, 2014). Importantly, these PFF-induced aggregates formed both *in vitro* and *in vivo* shared many features of α S aggregates in PD brains, including detergent insolubility, phosphorylation, ubiquitination, and filamentous in structure. Direct injection of α S PFFs

into the striatum of nTg mice has also been shown to induce α S aggregate formation, DA cell loss, and parkinsonism (Luk et al., 2012a). While these previous *in vivo* studies utilized direct, intracerebral, injection into target brain regions of interest, α S PFFs have also been demonstrated to induce nervous system pathology following peripheral injection (Sacino et al., 2014), demonstrating both the pathogenicity of fibrillar α S species, but also the versatility of the tool itself. Although there is an initial application of high amounts of exogenous α S in the PFF model that are above physiologic levels, the advantages are that this system does not require genetic modification of the cell or rodent, chronic overexpression of α S that may alter other cell biological processes and therefore confound observations and conclusions. Moreover, this model is species-specific as α S PFFs, but not monomers, are uniquely capable of inducing these pathological responses (Volpicelli-Daley et al., 2011, 2014). The α S PFF model affords both regional and temporal control as both injection and site are the main factors to consider, a contrast to transgenic models that constitutively express their transgene of interest.

Work investigating the mechanisms of α S PFF-mediated neurodegenerative processes has elaborated upon these initial observations, but one of the biggest concepts established thus far is the requirement for endogenous α S to mediate aggregate formation, toxicity, and transmission between neurons (Luk et al., 2012b; Mao et al., 2016; Volpicelli-Daley et al., 2011; Wu et al., 2019), demonstrating that PFFs likely exert their toxic and downstream effects through recruitment of endogenous α S. Uptake of α S PFFs occurs through endocytosis and requires transmembrane proteins such as LAG3 (Mao et al., 2016; Volpicelli-Daley et al., 2011). α S PFFs also exert synaptotoxic effects, including reduction of synaptic protein expression and network synchronization in an α S-dependent manner (Volpicelli-Daley et al., 2011), suggesting that α S PFFs exert these effects following internalization. At the level of the individual synapse, chronic α S PFF exposure

to primary neuronal cultures also alters spine dynamics, leads to loss of synapses, reductions in vesicle release, and excitotoxic effects such as calcium entry and oxidative stress (Dryanovski et al., 2013; Froula et al., 2018; Wu et al., 2019). Taken together, the versatility of the α S PFF represents an exciting tool for studying an array of pathological processes in PD and the contributions of pathogenic α S species to them.

Mechanisms driving cognitive decline in Parkinson's disease and dementia with Lewy bodies

The increasing disease burden exerted by PD and the α -synucleinopathies highlights the need to better understand disease mechanisms to better inform new treatments. In particular, cognitive dysfunction is a highly prominent and debilitating component of these diseases whose origins are not well established. Given that mutations in the *SNCA* gene are causal for PD, and α S-rich Lewy pathology is found in both sporadic and familial forms of the disease, as well as DLB, examining memory deficits through the lens of α S has led to promising insights.

Progression of PD and the onset of both motor and non-motor symptoms is thought to parallel the spread of Lewy pathology (LBs and LNs) from brainstem to midbrain to cortical regions (Braak et al., 2003). Neuropathologically, the distribution of LBs in the cortex and limbic system are hypothesized to contribute to cognitive deficits. Initial observations found that presence of cortical LBs is both highly sensitive and specific (>90%) for PDD, more than A β plaques or NFTs (Hurtig et al., 2000). Not surprisingly, the burden of cortical LB pathology positively correlates with severity of cognitive impairment and dementia, but it also inversely correlates with the duration between parkinsonism onset and dementia onset (Ballard et al., 2006). Given the arbitrary time distinction (1-year rule) between PDD and DLB, this finding may support placing dementia in PD and DLB as the same disease but on a continuum, potentially helping to simplify clinical management (Jellinger, 2018).

However, not all cases of PDD display cortical Lewy pathology, and not all patients with cortical LBs display dementia (Colosimo et al., 2003; Halliday et al., 2014). One possible explanation for this could be that LBs, especially in the context of cognitive dysfunction, may reflect the presence of pathogenic α S species and neuronal dysfunction in the cortex rather than a direct mediator of synaptic and cognitive deficits.

Upwards of 40% of PD patients at time of death have AD co-pathology, including A β plaques and tau NFTs, which may contribute to PDD and DLB (Irwin et al., 2013). Of interest, AD co-pathology is more prominent in PDD and DLB patients as compared to PD patients without dementia (Selikhova et al., 2009). Increased levels of AD co-pathology in PDD and DLB strongly correlate with increased rate of dementia onset and decreased survival (Compta et al., 2011; Irwin and Hurtig, 2018; Ruffmann et al., 2016), but this relationship has also been established in the context of cortical LB burden (Compta et al., 2011; Toledo et al., 2016), arguing for heterogeneity within this component of disease pathogenesis. In support, another study found that 28/51 of PDD cases had co-AD pathology (Sabbagh et al., 2009), yet the degree of cognitive impairment was clinically indistinguishable between the groups, suggesting that pathologic α S and cortical LB pathology may be a more critical determinant in driving deficits in PDD and DLB.

Instead of being an independent process in PDD and DLB, the high burden of AD co-pathology in the α -synucleinopathies may represent a synergistic process that underlies or even precipitates neuronal and cognitive dysfunction. Bigenic mice overexpressing both pathogenic human amyloid precursor protein (APP) and h α S developed memory and motor deficits prior to mice expressing only α S, had prominent presynaptic and cholinergic neurodegeneration, and also more intraneuronal α S inclusions as compared to single Tg animals (Masliah et al., 2001). Studies from another bigenic mouse model further this interaction: mice co-expressing h α S^{WT} and FTD-linked human mutant P301L tau

(SantaCruz et al., 2005) have increased presence of filamentous α S and tau inclusions in the same or separate neurons (Giasson et al., 2003). 3xTg-AD mice expressing human mutant APP, P301L tau, and PS1 crossed with M83 mice expressing h α S^{A53T} created a new mouse model that consequently exhibited accelerated cognitive decline and enhanced A β , tau, and α S pathologies as compared to the individual 3xTg-AD or M83 models (Clinton et al., 2010). More recent studies have continued to support this pathological interaction between α S and tau, with α S oligomers being capable of inducing tau oligomer production both *in vitro* and *in vivo* (Gerson et al., 2018; Greten-Harrison et al., 2010), and that antibody-mediated targeting of these tau oligomers prevented motor and cognitive deficits in M83 mice (Gerson et al., 2018). Another piece of evidence highlighting this relationship, the concept of driving neuronal dysfunction in the absence of overt neuropathology, is that the APOE4 genotype is associated with increased risk for dementia in the α -synucleinopathies, independent of A β plaque burden (Tsuang et al., 2013). Collectively, these findings, supported by clinical and neuropathological observations, directly implicate α S as a pivotal protein in mediating cognitive deficits in PDD and DLB, and that these pathogenic effects may be supported by interactions with other central neurodegenerative proteins like A β , and especially, tau.

From a structural standpoint, overt neurodegeneration is not a prominent feature in the α -synucleinopathies. Hippocampal sclerosis, particularly in CA1, is not a pervasive feature in PDD and DLB (Hely et al., 2008). Additionally, while reductions in hippocampal volume and gray matter are present in some cases, the severity of such reductions are less severe when compared to AD (Sanchez-Castaneda et al., 2009). More recently, hippocampal volume has become a tool for distinguishing causes of dementia, where preserved hippocampal volume in the context of memory deficits is a diagnostic feature of DLB and rules out AD (Kantarci et al., 2016). Indeed, hippocampal dysfunction, rather

than overt α S pathology or hippocampal loss, is a driver of cognitive deficits in PDD and DLB (Adamowicz et al., 2017; Hall et al., 2014). Although overt neuronal loss may not be observed in cortices or hippocampi of PDD/DLB patients, there are data suggesting synapse loss (Masliah et al., 1993; Schulz-Schaeffer, 2010). Along these lines, one line of TgWT mice displays α S aggregates at presynaptic terminals as well as a reduction in presynaptic terminals (Lim et al., 2011).

The hippocampus receives inputs from a variety of cortical and subcortical regions, and thus perturbation of these neurons and neurotransmitter systems may contribute to cognitive dysfunction. Degeneration of cholinergic neurons has been noted in PDD patients (Ballard et al., 2006, 2002; Zweig et al., 1993) and contribute to cognitive deficits, likely in similar fashion to that of AD. In this respect, loss of noradrenergic terminals has also been observed in h α S^{A53T} mice (Sotiriou et al., 2010). Within the cortex of PDD patients, decreases in cortical DA are observed without a loss of DAergic neurons (Takahashi et al., 2008). Synaptic plasticity (both long-term potentiation and depression) within the hippocampus has also been shown to be modulated by DAergic neurotransmission from the ventral tegmental area (VTA) and mesolimbic system through D1 and D5 receptors (Lemon and Manahan-Vaughan, 2006; Lisman and Grace, 2005; Wiescholleck and Manahan-Vaughan, 2014). It is therefore also conceivable that reduced DA levels and altered DA neuron function can lead to impaired hippocampal function. As such, while frank neurodegeneration within the hippocampus is not likely a primary driver of memory deficits in PDD and DLB, it is clear that altered neurotransmission within the hippocampus, likely a product of dysfunction and degeneration in other regions, contributes to this non-motor symptom.

Synaptic dysfunction is also likely contributor to cognitive decline in PDD and DLB. h α S^{WT} overexpression in mice promotes clathrin-mediated endocytosis of NMDARs from

postsynaptic sites, leading to reduced surface NMDAR expression and altered GluN2A/GluN2B NMDAR subunit ratios, ultimately producing impairment in LTP and memory (Chen et al., 2015; Cheng et al., 2011; Costa et al., 2012). Deficits in AMPA receptor (AMPA) function, another integral postsynaptic glutamatergic receptor, have also been implicated in α S-dependent deficits in mice overexpressing h α S^{A53T} (Teravskis et al., 2018). This study furthered the connection between neuropathologic observations in PDD and DLB and synaptic dysfunction, as h α S^{A53T}-mediated AMPAR deficits were associated with tau missorting to dendritic spines and in the absence of spine loss. Studies employing exogenous α S species have also advanced the role of pathogenic α S at the synapse. α S oligomers (α SOs) have been demonstrated to alter membrane permeability at the synapse through their capacity to induce pore formation (Pacheco et al., 2015; Tsigelny et al., 2012). Presynaptically, these α SOs can also impair vesicle recycling and reduce expression of key presynaptic proteins involved in this process such as SNAREs (Cheng et al., 2011; Scott et al., 2010; Volpicelli-Daley et al., 2011). At the postsynaptic surface, neurons exposed to α SOs display altered spine morphogenesis and stability, and altered NMDAR and AMPAR function that may lead to excitotoxic downstream events (Diogenes et al., 2012; Mattison et al., 2014). More recent studies have worked to establish a mechanism for α SO-mediated synaptotoxicity, which focus on pathogenic signal transduction via binding of α SOs to cellular prion protein (PrP^C) on postsynaptic surfaces, leading to a downstream cascade of signaling that involves adenosine receptors (A_{2A}) and metabotropic glutamate receptors (mGluR5) that ultimately produce increased NMDAR activation (via GluN2B phosphorylation) and excitotoxicity (Ferreira et al., 2015, 2017). Pharmacological inhibition of this pathway in TgWT mice restored synaptic and memory function, providing *in vivo* relevance to this pathway described via *in vitro* and *ex vivo* studies (Ferreira et al., 2017).

Studies in α S PFF-induced synaptic dysfunction support the major theme of α SO, that higher order, soluble, aggregates of α S are key drivers of pathological processes in PDD and DLB, in particular synaptic abnormalities underlying memory deficits. Exposure to α S PFFs leads to reductions in presynaptic proteins such as VAMP2 and SNAP25 in primary neuron cultures (Volpicelli-Daley et al., 2011), which may underly deficits in neuronal synchronization and neurotransmission as measured via mini excitatory postsynaptic current (mEPSC) frequency (Froula et al., 2018; Volpicelli-Daley et al., 2011; Wu et al., 2019). Similar to α SOs, α S PFF treatment *in vitro* also lead to spine abnormalities, including decreased density and amount of mature spines (Froula et al., 2018). However, an important distinction between α SO- and PFF-mediated impairments in synaptic plasticity likely result from their putative mechanisms. The current pathways defined in α SO-driven deficits suggest that α SO exert their toxic effects through both pre and postsynaptic mechanisms, but that postsynaptic abnormalities occur through receptor-mediated signal transduction. In contrast, α S PFF-mediated deficits appear to occur via cell-intrinsic mechanisms requiring recruiting and templating of endogenous α S to mediate their pathogenic effects. As such, it is evident that more studies are warranted to better delineate the intrinsic and extrinsic mechanisms driving these pathological processes. While is also clear that use of pathogenic α S species to model and investigate the mechanisms underlying PDD and DLB at the synaptic and neuronal levels is likely to provide significant insight into disease pathogenesis, it is also important to consider how these outputs are achieved in order to draw meaningful conclusions.

Extending beyond the individual synapse, dysfunction at the circuit or network level is also hypothesized to contribute to cognitive deficits in neurodegenerative diseases, most notably AD, but also PD (Palop and Mucke, 2016; Palop et al., 2006). Interestingly, h α S^{WT} overexpression in mice leads to a left shift in spectral power resembling slowing of

electroencephalogram (EEG) activity characteristic in DLB patients, as well as immunohistochemical alterations within the hippocampus indicative of aberrant network excitability (Morris et al., 2015). Long-term exposure primary neurons to α S PFFs *in vitro* lead to altered neuronal synchronization (coordinated spontaneous activity between neighboring neurons) as measured via calcium imaging (Volpicelli-Daley et al., 2011). Interestingly, this altered synchrony was partly explained via reduction in excitatory tone, and altered excitatory tone is a potential mediated of network-level dysfunction and aberrant activity *in vivo* (Palop and Mucke, 2010), potentially linking α S-mediated synaptic changes, network abnormalities, and cognitive dysfunction through final common mechanisms in different experimental models.

Hypotheses

The overarching objective of this thesis was to examine the mechanisms driving synaptic and memory deficits in α -synucleinopathies through several approaches. Considering the role of α S in presynaptic biology, abnormal α S, either mutated or aggregated, could be responsible for deficits in neurotransmission that underly PDD and DLB pathophysiology.

Additionally, given the contributions of other neurodegenerative disease-relevant proteins to neuropathology and cognitive decline in PDD and DLB, it is hypothesized that α S-mediated memory deficits may be produced via interactions with other proteins such as tau. However, prior to this work, it remained unknown if α S mediates cognitive decline *in vivo* and tau contributes to α S-mediated behavioral deficits. Through use of a transgenic mouse model of α -synucleinopathy (TgA53T), the mechanisms of h α S^{A53T}-mediated cognitive decline were first examined, with the hypothesis that tau-mediated postsynaptic deficits were critical for this process. In addition to characterizing the progressive nature

of α S-mediated synaptic and behavioral deficits in TgA53T mice, TgA53T mice lacking endogenous mouse tau expression were generated to directly test the role of tau in this process. To determine the cellular and molecular mechanisms underlying α S-mediated memory deficits in TgA53T mice, biochemical, immunohistochemical, and electrophysiological approaches were employed to examine changes in protein expression and function. While the TgA53T model represents a valuable model for investigating how α S abnormalities lead to pathological processes in α -synucleinopathies, the majority of PD cases are sporadic, without known genetic mutations in *SNCA*. As such, the second major hypothesis was if pathogenic species of aggregated h α S^{WT}, the α S PFF, were capable of driving cellular, molecular, and physiological abnormalities in a similar pathway to that of TgA53T, asking the question if pathogenic α S, either h α S^{WT} or α S PFFs, produced synaptic impairments through common mechanisms that could collectively represent a final common pathway for deficits in synaptic deficits that ultimately underly memory deficits.

Authorship

The studies and findings in this thesis represent the product of excellent collaborations and support from a variety of individuals and laboratories. I am grateful for experimental, technical, and scientific support and efforts they provided in support of my research and graduate training. Contributions are also noted in the figure legends.

For all mice used in Chapters 2 and 3, animal husbandry was done with the support of Héctor Martell-Martinez (HMM).

Chapter 2: Behavioral studies were conducted in collaboration with Christopher Gallardo PhD (CG) and Michael A. Benneyworth PhD (MAB) of the UMN Mouse Behavioral Core. Electrophysiological studies in acute hippocampal slices were performed by Ana Covelo PhD (AC) and Carmen Nanclares PhD (CN) in the lab Alfonso Araque. Dissociated hippocampal neuronal culture electrophysiology was performed with the guidance of Peter J. Teravskis (PJT) in the lab of Dezhi Liao PhD. Dot blot and Fyn biochemical analyses were performed by Mathew A. Sherman (MAS) and Sylvain E. Lesné PhD (SEL) of the Lesné lab. Immunohistochemical studies, including brain sectioning, were accomplished with the support of Emmanuel Okematti (EO) and Joyce Meints (JM). Confocal imaging was performed in the UMN University Imaging Centers thanks to the teaching and support of Mark A. Sanders PhD. Preparation and injection of recombinant α S pre-formed fibrils were conducted with the support of HMM.

Chapter 3: Preparation of recombinant α S monomers and α S pre-formed fibrils was conducted by HMM. Dissociated hippocampal neuronal culture imaging and electrophysiology were performed with the guidance of PJT.

Chapter 2: Tau is required for progressive synaptic and memory deficits in a transgenic mouse model of α -synucleinopathy

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Introduction

Parkinson's disease dementia (PDD) and Dementia with Lewy Bodies (DLB) are closely related diseases in the α -synucleinopathy family that comprise the second most common neurodegenerative dementia (Jellinger and Korfczyn, 2018). These and other α -synucleinopathies are characterized by the presence of cytoplasmic inclusions termed Lewy bodies (LB) and Lewy neurites (LN), composed primarily of fibrillar α -synuclein (α S) (Spillantini et al., 1998b). In addition to motor dysfunction arising from the loss of dopaminergic neurons in the substantia nigra pars compacta (Obeso et al., 2010), the broad distribution of LB/LN across multiple neuronal populations has led to an understanding in PD that the disease extends beyond the basal ganglia (Aarsland et al., 2008, 2017). Dementia in the α -synucleinopathies is hypothesized to be a product of α S abnormalities at cortical and hippocampal synapses. This pathogenic role for α S is supported by genetic and pathological observations: mutations in the SNCA gene encoding α S are causative for early-onset, familial autosomal dominant forms of PD (Houlden and Singleton, 2012; Poewe et al., 2017; Polymeropoulos et al., 1997), and LBs and LNs are found in both familial and sporadic cases of PD (Spillantini et al., 1997). While degeneration of cortical and hippocampal neurons is not a significant feature of PDD and DLB (Hall et al., 2014), cortical and hippocampal α S pathology show significant correlation with dementia (Adamowicz et al., 2017; Colom-Cadena et al., 2017; Hall et al., 2014;

Harding and Halliday, 2001; Jellinger and Korczyn, 2018; Spillantini et al., 1998b). Because α S is a cytosolic protein enriched at presynaptic terminals with established roles as an inhibitor of neurotransmitter release (Abeliovich et al., 2000; Nemani et al., 2010) and a presynaptic chaperone (Burré et al., 2010, 2013, 2014; Chandra et al., 2005), it is hypothesized that disease-associated α S may cause memory deficits through mechanisms involving presynaptic dysfunction. We recently showed that mutant A53T human α S ($h\alpha S^{A53T}$) expression causes deficits in learning, memory, and synaptic plasticity in mice (Teravskis et al., 2018). Significantly, while we show that presynaptic deficits, characterized by decreased probability of neurotransmitter release, are present in both wild-type and mutant human α S expressing neurons, only $h\alpha S^{A53T}$ expression caused defects in postsynaptic function and synaptic plasticity. Mechanistically, this unique $h\alpha S^{A53T}$ -induced postsynaptic dysfunction is mediated through a process involving tau: GSK3 β -dependent tau phosphorylation, subsequent tau missorting to dendritic spines, and calcineurin-dependent AMPA receptor (AMPA) internalization. These deficits in neurotransmission appear in the absence of overt neuropathology, suggesting that neuronal dysfunction is not a consequence of neurotoxicity and neurodegeneration.

In this study, we sought to extend our prior findings by mechanistically connecting our *in vitro* studies with the memory deficits *in vivo*. To accomplish this, we directly tested whether tau expression was required for α S-induced cellular, physiological, and behavioral deficits in the TgA53T mouse model of α -synucleinopathy. We show that TgA53T mice exhibit progressive memory deficits associated with the presence of postsynaptic, but not presynaptic, deficits. More important, we demonstrate that loss of tau completely reversed the onset of memory deficits in multiple experimental paradigms and that tau is required for α S-mediated neurophysiological deficits, including postsynaptic dysfunction, impairments in glutamatergic neurotransmission, and short- and

long-term plasticity. Significantly, these parameters are independent of any α S pathology or neurodegenerative changes. Finally, the onset of neurophysiological and memory deficits coincides with the onset of seizure-like network hyperactivity. We propose that early tau-dependent postsynaptic deficits caused by mutant α S is mechanistically linked to the onset of network abnormalities and memory deficits. Our results provide novel insights on how pathological α S precipitates impairments in neurotransmission and memory loss and may inform the development of new therapeutic approaches for PDD and DLB.

Materials and methods

All animal studies were performed in accordance with the NIH guidelines for the use of animals in research and approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Experimental group sizes (n) are reported in each figure.

Transgenic A53T α -Synuclein mutant and tau-knockout mice

We bred four key genotypes for this study. TgA53T animals contain a transgene expressing the human mutant A53T α -synuclein (α S, h α S^{A53T}: line G2-3). All mice were maintained in the C57BL/6J background strain (stock 0006644, Jackson Labs; Bar Harbor, ME). TgA30P animals express human mutant A30P α S (h α S^{A30P}: line O2, TgA30P) at similar levels to TgA53T, and TgWT animals express human wild-type α S (h α S^{WT}: line I2-2, TgWT), but at levels lower to TgA53T and TgA30P. Transgene expression for all animals is controlled by the mouse prion promoter (MoPrP) (Lee et al., 2002). TgA53T, TgA53T/mTau^{-/-}, TgA30P, and TgWT were all heterozygous for their respective h α S transgene. Non-transgenic (nTg) controls came from within these litters. To generate transgenic animals expressing h α S^{A53T} (TgA53T) and lacking endogenous mouse tau

(mTau^{-/-}), TgA53T males were bred to Mapt^{tm1(EGFP)Klt/J} females (stock 004779, Jackson Labs) (Tucker et al., 2001) in successive generations to generate offspring that lacked endogenous mouse tau expression and with, or without, hαS^{A53T} transgene expression. Transgenic progenies were identified by PCR analysis of tail DNA as previously described (Lee et al., 2002). Because all mice were maintained on the C57BL/6J background, we were able to generate TgA53T and nTg cohorts separate from the TgA53T/mTau^{-/-} and mTau^{-/-} cohorts. This approach was used because the use of heterozygous mice for breeding leads to a very low yield of animals with the experimentally-desired genotype (25%), increased heterogeneity between littermates, and more importantly, a larger spread in birthdates and consequently ages of mice, introducing potential methodological variations. Additionally, this low yield of experimentally-desired mice would translate into a large population of unused, “wasted”, animals, something we consciously worked towards avoiding. Generating TgA53T/mTau^{+/+} (TgA53T) and TgA53T/mTau^{-/-} cohorts via independent homozygous matings produced progeny within a tighter age range and allowed us to use 100% of the offspring for experiments. A breeding schematic and diagram elaborating the rationale can be found in **Figure 2.1**.

The TgA53T (G2-3) mouse model exhibits an adult-onset hyperactive locomotor phenotype (Unger et al., 2006) prior to the onset of motor dysfunction. This motor dysfunction phenotype rapidly progresses from slight ataxic signs (gait and balance abnormalities) to complete paralysis within 2-3 weeks (Lee et al., 2002). This pattern of motor deficit is also observed in another transgenic mouse model expressing hαS^{A53T} under control of the mPrP promoter (M83) as rotarod performance declines only following sudden and dramatic disease onset (Giasson et al., 2002). Since the initial characterization of TgA53T mice, the average age of disease onset has drifted from approximately 10 to 13 months of age (Lab of Michael K. Lee, unpublished data).

Neuropathologically, the onset of motor symptoms in mPrP-h α S^{A53T} transgenic mice (G2-3 and M83) is associated with the presence of α S pathology in subcortical areas (midbrain, brain stem, and spinal cord) but not in forebrain regions including the hippocampus and amygdala (Giasson et al., 2002; Lee et al., 2002). Any animals showing overt motor symptoms were excluded from further analysis.

Male mice were exclusively used for all behavior studies. For the electrophysiological, biochemical, and immunohistochemical analyses, both males and females were used. In these studies, efforts were made to use equal numbers of mice from each sex within each genotype cohort.

All mice in the colony were kept under specific pathogen-free (SPF) conditions in a 14 hours (hr) light/10 hrs dark cycle, and had free access to food and water. All housing and experimental protocols involving mice were conducted with strict adherence to the National Institutes of Health (NIH) Animal Care and Guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota.

Dissociated neuronal culture electrophysiology

Dissociated neuronal cultures were established from harvested from mouse hippocampi postnatal day 0 or 1 (P0-P1) as previously described (Teravskis et al., 2018) with modifications. Briefly, mouse hippocampi were dissected out and stored individually by pup in Hibernate A media (Brain Bits; Springfield, IL) at 4°C. While stored, each pup from the cultured litter was genotyped. Cerebellar tissue was used for genomic DNA isolation via QuickExtract DNA (Epicentre-Lucigen; Madison, WI) and genotyping of pups was performed immediately after hippocampal isolation. Following genotype identification, hippocampi of identical genotypes were pooled, dissociated, and plated on onto 35 mm

μ -dishes containing a poly-D-lysine and laminin-coated polymer coverslip (Ibidi; Fitchburg, WI). 24 hrs after initial plating, media was removed and NbActiv4 growth media was added (Brain Bits). NbActiv4 media was refreshed every 3-4 days by removing 1 mL media in the dishes and adding 1 mL fresh media. For all procedures and media exchanges, solutions were equilibrated in a tissue culture incubator (37°C, 5% CO₂) for at least 2 hrs prior to use. The age of *in vitro* dissociated cultured hippocampal neurons began with the day of initial plating, and each day that followed was counted as one day *in vitro* (DIV). All experiments were performed on neurons from at least 3 independent cultures with a minimum of 5 animals per culture. Miniature excitatory postsynaptic currents (mEPSC) were recorded from cultured dissociated mouse hippocampal neurons at 21–25 DIV with a glass pipette (resistance of ~5 M Ω) as previously described (Teravskis et al., 2018). Recordings ranged from 3-15 minutes (min) and stable traces longer than 1 min in duration were analyzed. All mEPSCs >2 pA were manually counted with MiniAnalysis (Synaptosoft Inc; Fort Lee, NJ). Each mEPSC event was visually inspected and only events with a distinctly fast-rising phase and a slow-decaying phase were accepted. Relative cumulative frequencies were derived from individual events and the averaged parameters from each neuron were treated as single samples in any further statistical analyses.

Acute hippocampal slice electrophysiology

Acute coronal hippocampal slices (approximately 350 μ m thick) were obtained from two age windows of mice (2-3 months and 5-6 months) from nTg, TgA53T, TgA53T/mTau^{-/-}, and mTau^{-/-} animals utilizing well-established methods (Teravskis et al., 2018).

Briefly, slices were incubated in ACSF (containing in mM: NaCl 124, KCl 5, NaH₂PO₄ 1.25, MgSO₄ 2, NaHCO₃ 26, CaCl₂ 2, glucose 10; gassed with 95% O₂, 5% CO₂, and maintained between pH 7.3 and 7.4) at room temperature for at least 1 hr before use. For

studies, slices were transferred to an immersion recording chamber, superfused at 2 mL/min with oxygenated ACSF and visualized under an Olympus BX50WI microscope (Olympus Optical; Japan). In order to study excitatory postsynaptic currents (EPSCs), picrotoxin (50 μ M) and CGP54626 (1 μ M) were added to the solution to block GABA-A and GABA-B receptors, respectively. Whole-cell electrophysiological recordings were obtained from hippocampal CA1 pyramidal neurons using patch electrodes (3-10 M Ω) filled with an internal solution containing in mM: cesium gluconate 117, HEPES 20, EGTA 0.4, NaCl 2.8, TEA-Cl 5, ATP 2, GTP 0.3, and kept at pH 7.3. Recordings were obtained with PC-ONE amplifiers (Dagan Instruments; Minneapolis, MN). Membrane potentials were held at -70 mV. Signals were filtered at 1 kHz, acquired at a 10 kHz sampling rate, and fed to a Digidata 1440A digitizer (Molecular Devices; San Jose, CA). pCLAMP 10.4 (Axon Instruments, Molecular Devices; San Jose, CA) was used for stimulus generation, data display, data acquisition, and data storage. To record evoked EPSCs, theta capillaries filled with ACSF were used for bipolar stimulation and placed in the stratum radiatum to stimulate Schaffer collaterals. Input-output curves of EPSCs were made by increasing stimulus intensities from 0 to 100 μ A. Paired pulse facilitation was done by applying paired pulses (2 millisecond (msec) duration) with 25, 50, 75, 100, 200, 300, and 500 msec inter-pulse intervals. The paired pulse ratio was calculated by dividing the amplitude of the second EPSC by the first ($PPR = EPSC-2 / EPSC-1$). Synaptic fatigue was assessed by applying 15 consecutive stimuli in 25 msec intervals. AMPA currents were obtained at a holding potential of -70 mV. NMDA currents were obtained at a holding potential of + 30 mV. To ascertain the AMPA to NMDA receptor current ratio the NMDA component was measured 50 msec after the stimulus, when the AMPA component had decayed. Recordings of miniature EPSCs (mEPSCs; $V_h = -70$ mV) were made in the presence of tetrodotoxin (TTX; 1 μ M) in addition to the respective solution. For long-term

potentiation (LTP), CA1 pyramidal neurons first underwent a baseline recording of 10 mins followed by tetanic stimulation in the Schaffer collaterals (4 trains at 100 Hz for 1 second (sec); 30 sec intervals). After LTP induction, neurons were recorded for 50 mins. The presence of LTP was determined by comparing the average EPSC amplitudes from last 5 mins of pre-stimulus baseline recording to the average EPSC amplitudes from the last 5 mins post-stimulus recordings. All experiments were performed at room temperature.

Behavioral testing

Behavioral testing was performed with the support and guidance of the Mouse Behavior Core at the University of Minnesota. ANY-maze software (Stoelting Co.; Wood Dale, IL) was used in conjunction with paired video cameras to track and record animal movements during all behavioral testing, and all subsequent analysis. Behavior experiments, unless dictated by testing protocols, were not performed on consecutive days, giving animals at least two days to rest between experiments. Mice were also habituated to handling prior to testing. Animals from all genotypes were tested together. Each behavioral age point came from a separate cohort. Additionally, all groups tested, including those at different age points, went through the battery of tests in the same order.

Open field

Locomotor activity was assessed using open field testing. Animals were placed individually into a square 40 cm x 40 cm arena with white, opaque walls and flooring, lit to 200 lux. Movement was video-recorded for 60 mins and began immediately after placing the animal in the open field testing chamber. After the test, animals were returned to their home cage.

Barnes maze

Spatial learning and memory were assessed via Barnes maze (San Diego Instruments; San Diego, CA), following established protocols (Barnes, 1979; Sunyer et

al., 2007). The maze itself consists of a white plastic circular top with 20, evenly-sized and evenly-spaced holes around the perimeter. Spatial cues were placed on all four walls of the behavioral testing room, lit to 300 lux during testing. Mice were acclimatized to the testing room for 30 mins at the beginning of each training session. Training days consisted of 4 trials per day for 4 days. Training trials ended with the subject climbed into the escape box within the goal quadrant located under 1 of the holes or when the maximum trial duration of 180 sec was reached. Upon entering the escape box during training, room lights turned off and animals remained in the escape box for 60 sec before returning to their home cage in the testing room to await the next trial. While the platform was rotated between each trial on each day (to obscure the impact any animal scents or non-spatial cues on the maze), the location of the escape box and goal quadrant relative to the room remained constant during all training trials and days. Subjects were run in small groups of 6 mice or less so that no more than 20 mins passed between trials for a given animal during training. On the day following the last training trial, memory was assessed in single 90 sec probe trial tests where the target escape box in the goal quadrant was replaced with a false box cover identical to the other 19 holes, and the exploration pattern of each subject was examined.

Y maze

Short term spatial learning and memory was assessed via Y maze. Animals underwent 30 mins acclimation periods in the testing room prior to testing. Cues were visible on walls of the testing room as well as within the maze, at the end of each arm (extra-maze and intra-maze, respectively). The Y maze was constructed out of opaque white plastic and located at the center of the testing room lit to 150 lux. During the first of two phases, only two of the three arms were available for exploring during the 10 mins learning trial: the start arm and the familiar arm. For all trials, animals were placed at the end of the start arm, furthest point from center. During the 60 mins inter-trial interval between learning and

recognition, animals returned to their home cage but remained in testing room. The 5 mins recognition trial was then performed, with all 3 arms were available for exploration: start, familiar, and novel. The Y maze was also cleaned between each trial/animal to avoid confounds due to scent.

Context Fear Conditioning

Context fear conditioning was conducted using the Near Infrared (NIR) Video Fear Conditioning package for mice (Med Associates, Inc; Fairfax, VT). A context conditioning environment (specific olfactory, tactile, and visual elements) is paired with foot shocks on the first day: a 10 mins context conditioning trial was first performed in the NIR fear conditioning chamber: electrified metal bars on the floor, providing 0.7 V foot shocks for 2 sec 5 times per trial at variable intervals averaging 90 sec between shocks, squared off corners and metal walls, and a 33% Simple Green solution for scent. 24 hrs following conditioning animals were underwent the context memory trial where freezing was assessed for 3 min with identical chamber conditions to the conditioning trial except no foot shocks (Similar). If the animal successfully learns the context-shock association, re-exposure to the context environment 24 hrs post-conditioning, the “similar” setting, will elicit fear in the animal placed in that environment. 2 hrs after the similar trial, mice were placed in the chamber for the novel context trial (Novel), white, solid plastic flooring and curved walls with a stripe design inside, and a 0.5% vanilla solution for scent, to show the context specificity of the fear memory.

Western blot analyses

Chemiluminescence Western Blotting

At predefined age points, mice were harvested and brain regions (cortices and hippocampi, in particular) were dissected out and stored at -80°C. To extract proteins, cortical and hippocampal samples from -80°C were first thawed at -20°C overnight before

being weighed and homogenized via Dounce homogenization in 10 volumes of TNE solution in mM: Tris-HCl 50, NaCl 150, and EDTA 5. Following homogenization, an equal volume of Complete TNE was added: TNE plus 0.5% NP40, 0.5% DOC, 1% SDS, and HALT protease and phosphatase inhibitors (Thermo Fisher Scientific; Waltham, MA). Samples were then spun down at 16,000 g for 90 mins at 4°C. Supernatant was collected and subjected to immunodepletion via serial incubations with Protein A and G Mag Sepharose beads (GE Life Science; Pittsburgh, PA) rocking at 4°C for 60 mins. Protein concentration was assessed via BCA assay (Pierce, Thermo; Rockford, IL). Samples were prepared to equal concentration in reducing, SDS-sample, Laemmli buffer (Boston BioProducts; Ashland, MA). For western blot analysis, protein lysates were run on Criterion™ TGX™ gels (BioRad; Hercules, CA) and transferred onto nitrocellulose membranes. Membranes were then blocked using 5% non-fat dry milk in tris-buffered saline (TBS) with 0.1% Tween 20 (TBSTw) for 1 hr at room temperature (RT). Primary antibodies were diluted in OneBlock solution (Genesee Scientific; San Diego, CA) and incubated on membranes overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen; Carlsbad, CA) were diluted in 5% non-fat dry milk in TBSTw and incubated on membranes 1 hr at RT. Membranes were then developed using chemiluminescent substrates (BioRad and Thermo) and the ImageQuant LAS 4000 detection system (GE Life Sciences). Densitometry on western blot images were subsequently analyzed using ImageQuant TL 8.1 software (GE Life Sciences).

Fluorescence Western Blotting

SDS-PAGE was performed on precast 10.5–14% Criterion™ Tris-Tricine gels, 10.5–14% or 4–10.5% Tris-HCl gels (Bio-Rad). Protein levels were normalized by using 10-100 µg of protein per sample (depending on the targeted protein). The samples were

resuspended with 4x Tricine loading buffer and boiled for 5 mins before loading. Proteins were transferred onto 0.2- μ m nitrocellulose membrane (Bio-Rad) following electrophoresis. Membranes were blocked in 5% BSA (Sigma; St. Louis, MO) in TBSTw for 1–2 hrs at room temperature and probed with the appropriate antisera/antibodies diluted in 5% BSA in TBSTw. Primary antibodies were probed with secondary antibodies conjugated with infrared dyes (LI-COR Biosciences; Lincoln, NE). Densitometry analyses were performed using the Odyssey software (LI-COR Biosciences). Normalization was performed against GAPDH. Quantification by software analysis was performed as described previously (Amar et al., 2017; Khan et al., 2018; Larson et al., 2012, 2017; Sherman et al., 2016).

Dot blot analyses

Protein isolates were obtained from samples at the same time they were being prepared for western blotting. Samples diluted to identical concentrations (1 μ g/ μ L) and 2 μ g of sample was adsorbed onto a nitrocellulose membrane and air dried for 30 mins. Following activation in 10% methanol (in TBS), membranes were blocked in TBS containing 5% bovine serum albumin (BSA) for 30 mins at room temperature (RT). Samples were then incubated in primary antibody overnight at 4°C in 5% BSA in TBS. Following primary antibody incubation and TBS washes, secondary incubation was performed for 1 hr at RT in the dark in 5% BSA in TBS with IRDye secondary antibodies (anti-mouse IgG-IR800 at 1:100,000 and anti-rabbit IgG-IR680 at 1:150,000) (LI-COR Biosciences). Membranes were then washed, imaged and analyzed using the LI-COR Odyssey imaging system.

Insoluble and Soluble Protein Extraction

An equal volume of 2% Triton™ X-100 (Sigma-Aldrich; St. Louis, MO) in TNE was added to the Dounce homogenized lysate in TNE, mixed, and then sonicated for 30 sec at 4°C. Samples were then spun down at 20,000 g for 60 mins at 4°C. The supernatant from this spin was saved as the soluble fraction. The pellet was saved as the insoluble fraction, washed in 1% Triton™ X-100 in TNE and spun down again. Complete TNE was added to both soluble and insoluble fractions. Fractions were then sonicated, boiled for 10 mins at 95°C, spun down at 16,000 g for 15 mins at 4°C, and then the supernatant from each fraction was saved. Protein concentration was assessed via BCA assay (Pierce, Thermo; Rockford, IL). Samples were then prepared for western blotting identically to the “Chemiluminescent Western Blotting” protocol. After transfer, membranes were stained with 0.1% Ponceau S solution (Sigma-Aldrich) to confirm equal protein loading and transfer. Samples then underwent primary and secondary antibody incubation and chemiluminescent detection following “Chemiluminescent Western Blotting” methods.

Immunohistochemical analyses

At predefined age points, mice were anesthetized with isoflurane and euthanized via transcardial perfusion with saline. Animals were first perfused with ice cold K-free PBS, containing in mM: NaCl 13, Na₂HPO₄•2H₂O 0.7, and NaH₂PO₄•2H₂O 0.3, and adjusted to pH 7.2. Following saline perfusion, animals were then perfuse-fixed with 4% paraformaldehyde (PFA). Brains were then isolated and stored in 4% PFA at 4°C for an additional 48 hrs before being transferred to a 30% sucrose solution in PBS prior to slicing. After a minimum of 72 hrs in sucrose solution, brains were sliced coronally at a thickness of 30 μM via HM 450 sliding microtome (Thermo Fisher Scientific) and stored in a cryoprotectant solution (30% sucrose, 30% ethylene glycol in PBS) prior to immunostaining. Immunofluorescent staining was preformed utilizing the Pelco BioWave

Plus (Ted Pella; Redding, CA) and mounted on slides for imaging. Prior to staining sections were rinsed in TBS, permeabilized with 0.1% Triton X-100 in TBS and blocked in 50% Background Sniper solution in TBS (Biocare Medical; Pacheco, CA). Primary and secondary antibodies were diluted to target concentrations in 5% Background Sniper (Biocare Medical) in TBSTw and allowed to incubate on sections in the Pelco BioWave for 1.5 hrs each. Alexa Fluor (Abcam; Cambridge, MA) or Alexa Fluor Plus (Thermo Fisher Scientific) secondary antibodies were used as dictated by primary antibody host. If required, sections were then 4',6-diamidino-2-phenylindole (DAPI)-counterstained (Thermo Fisher Scientific). All sections were treated with TrueVIEW™ (Vector Laboratories; Burlingame, CA) for autofluorescence quenching and mounted on slides for future imaging. Confocal microscopy images were acquired with the Nikon C2 Confocal Microscope System (Nikon; Melville, NY). Imaging processing and analysis were performed using NIS-Elements software (Nikon). Staining, imaging, and analysis were performed in collaboration with the University of Minnesota's University Imaging Centers on the Twin Cities campus. Analysis and quantification of biomarkers of neuronal network hyperexcitability (c-Fos, neuropeptide Y (NPY), and calbindin) were performed as described previous (Palop et al., 2011).

Preparation and peripheral injection of human wild-type α -synuclein pre-formed fibrils (h α S^{WT} PFFs)

Recombinant full length human wild-type α S pre-formed fibrils (PFFs) were generated following the protocol established by Volpicelli-Daley et al., 2014. α S PFFs were generated by first incubating purified recombinant monomeric α S at 5 mg/mL in phosphate-buffered saline (PBS) at 37°C with continuous shaking for 7 days via ThermoMixer (Eppendorf; Hamburg, Germany). During this shaking process, the

presence of amyloid α S fibrils production was assessed and confirmed using Thioflavin T fluorometry. Following shaking and saturation of Thioflavin T fluorescence levels, α S PFFs were subsequently aliquoted and stored at -80°C . The generation of α S PFFs and their seeding capacity were confirmed through sedimentation assay, subjecting an aliquot of PFFs to ultracentrifugation, and assessing the amount of α S in pellet and supernatant via SDS-PAGE (BioRad) and Coomassie Brilliant Blue (Thermo Fisher Scientific) staining. For peripheral injections into mice, 5 mg/mL α S PFF aliquots in PBS were taken from -80°C , thawed at room temperature, and diluted to a working concentration of 0.25 mg/mL (250 $\mu\text{g}/\text{mL}$) in PBS. α S PFFs at this concentration were then sonicated utilizing a Fisher Scientific Branson micro probe tip sonicator (Fischer Scientific; Hampton, NH) with 60 pulses at 20% power for a total of 120 sec, 1 sec “on”, 1 sec “off”. Following sonication, PFFs were ready for injections via Hamilton microliter syringe (Hamilton Company; Reno, NV). For injections, mice were lightly anesthetized using isoflurane and 5 μg of α S PFFs (50 μL of 250 $\mu\text{g}/\text{mL}$ α S PFFs) were injected into the *biceps femoris muscle* bilaterally at rate of approximately 10 μL per sec, for a total of 10 μg α S PFFs per animal. To control for any between-batch differences of PFFs, all mice used for this study were injected on the same day with a common, single batch of prepared α S PFFs. Controls within each group were injected with 50 μL of PBS bilaterally.

Antibodies

A detailed list of all antibodies used for experiments and studies here can be found in **Table 2.1**.

Experimental design and statistical analyses

All statistical analyses were performed in Prism 8.1.0 (GraphPad Software; San Diego, CA). Data visualization and presentation were performed via Prism (GraphPad) and JMP 14 (SAS; Cary, NC). For parametric data: one-, two-, and three-way ANOVAs were utilized for one, two, and three variable analysis on multiple groups, respectively; t tests were also performed when analyzing only two groups. For nonparametric data: Kruskal-Wallis one-way ANOVA for three or more groups; Mann-Whitney test for two groups. Posthoc analyses were performed on all data that were significantly different. Welch's correction, Sidak's posthoc, and Tukey's posthoc analyses were used due to considerations for within-group variances and sample size. The Geisser-Greenhouse correction was used for two- and three-way repeated measures ANOVAs due to considerations for sphericity. For all, statistical significance was set for $\alpha = 0.05$. Data representations are described in figure legends

Abbreviation	Line	Expression	Reference
TgA30P	O2	Human missense mutant A30P α S	Lee et al., 2002, <i>PNAS</i>
TgA53T	G2-3	Human missense mutant A53T α S Available via Jackson Labs: Tg(Prnp-SNCA*A53T)23Mkle, strain 006823	
TgWT	I2-2	Human wild-type α S	

Table 2.1 – List of transgenic mouse lines used in experiments.

Loading Controls	Company	Reference	Use
Actin	Sigma-Aldrich	A2066	WB
GAPDH (GA1R)	Invitrogen	MA5-1738	WB
GAPDH (D16H11)	Cell Signaling	5174	WB
α -Synuclein Species	Company	Reference	Use
α -Synuclein (total)	BD Transduction	610787	WB
4D6 (total)	BioLegend	834304	DB
Phospho-serine 129 α S (pS129 α S)	Abcam	Ab51253	WB

F8H7 (conformation-specific oligomers)	Dr. Rakez Kayed (gift)	Sengupta et al., 2015	DB
FILA-1 (fibrillar α S)	Dr. Poul Jensen (gift)	Paleologu et al., 2009	DB
LB509 (human)	BioLegend	807707	DB
MJFR-14-6-4-2 (MJFR14, conformation-specific)	Abcam	Ab209538	DB
HuSyn1 (human)	Dr. Michael K. Lee	Lee et al., 2002	WB
Syn33 (conformation-specific oligomers)	Dr. Rakez Kayed (gift)	Sengupta et al., 2015	DB
Tau Species	Company	Reference	Use
Tau5 (total)	Millipore	MAB361	WB
AT8 (phospho-Ser-202 and -Thr-205)	Invitrogen	MN2010	WB
A11 (tau and α S oligomeric species)	Dr. Rakez Kayed (gift)	Khan et al., 2018	DB
CP13 (phospho-Ser-202)	Dr. Peter Davies (gift)	Khan et al., 2018	WB
OC (tau and α S amyloid fibrils)	Dr. Rakez Kayed (gift)	Khan et al., 2018	DB
PHF1 (phospho-Ser-396 and -Ser-404)	Dr. Peter Davies (gift)	Khan et al., 2018	WB
T22 (tau oligomers)	Dr. Rakez Kayed (gift)	Lasagna-Reeves et al., 2012	DB
Neuron and Synapse Markers	Company	Reference	Use
β -Synuclein	Thermo Fisher	PA5-25738	WB
MAP2	Novus Biologicals	NB300-213	IF
NeuN	Abcam	Ab177487	IF
PSD95	Abcam	Ab2723	IF
PSD95	Cell Signaling	3450	IF, WB
Synapsin 1/2	Synaptic Systems	106-002	WB
Synaptophysin	Abcam	Ab32127	IF, WB
Glutamate Receptors	Company	Reference	Use
GluA1 (D4N9V)	Cell Signaling	13185	WB
GluA2/3	Millipore	07-598	WB
GluN1	BD Pharmingen	556308	WB
GluN2A	Cell Signaling	4205	WB
PrP-Fyn-GluN2B	Company	Reference	Use
Fyn (FYN-01)	Abcam	Ab1187	WB
pTyr417-Src	Cell Signaling	2101	WB
GluN2B (D15B3)	Cell Signaling	4212	WB

pTyr1472-GluN2B	Cell Signaling	4208	WB
PrP (6D11)	BioLegend	808004	WB
PrP (8B4)	Santa Cruz	sc-47729	WB
Network Markers	Company	Reference	Use
Calbindin (D114Q)	Cell Signaling	13176	IF
c-Fos	Synaptic Systems	226-003	IF
Neuropeptide Y (D75YA)	Cell Signaling	11976	IF

Table 2.2 – List of antibodies utilized in experiments. DB: Dot blot. IF: Immunofluorescence. WB: Western blot.

Results

Mutant α -synuclein-dependent cognitive dysfunction in spatial learning and memory is both progressive and tau-dependent

Several α -synuclein (α S) transgenic mouse models display memory deficits (Chesselet et al., 2012; Lim et al., 2010, 2011; Magen et al., 2012), including the model expressing human mutant A53T α S (TgA53T) (Chesselet et al., 2012; Lim et al., 2010, 2011; Paumier et al., 2013; Teravskis et al., 2018). However, the mechanistic basis for cognitive impairment in these mouse models is poorly defined. Previously, we established a novel connection between human mutant A53T α S ($h\alpha S^{A53T}$) and postsynaptic impairments in AMPAR function that require tau phosphorylation and mislocalization to dendritic spines (Teravskis et al., 2018). Because these glutamatergic postsynaptic impairments are linked to cognitive dysfunction (Hoover et al., 2010), we hypothesized that cognitive deficits in TgA53T mice are tau-dependent. To directly test the requirement of tau in mediating α S-induced memory loss, we generated TgA53T mice lacking endogenous mouse tau expression by breeding TgA53T mice to the mouse tau knockout mice ($mTau^{-/-}$) (Tucker et al., 2001). The four genotypes (TgA53T, TgA53T/ $mTau^{-/-}$, $mTau^{-/-}$, and non-transgenic (nTg) littermate controls) appear to develop and age normally for the duration of the study (**Figure 2.1**).

Previously, we showed that TgA53T mice exhibit spatial memory deficits in the Barnes maze (BM) (Barnes, 1979; Sunyer et al., 2007) at 12 months of age (Teravskis et al., 2018), however, it is unknown if general overexpression of $h\alpha S$ also leads to memory deficits. To confirm that only TgA53T mice exhibit memory deficits in our experimental conditions, we examined whether TgWT ($h\alpha S^{WT}$) and TgA30P ($h\alpha S^{A30P}$) mice in our colony exhibit deficits in spatial learning and memory at older ages. TgA53T and TgA30P mice have similar human mutant α S transgene expression levels, while TgWT mice display

lower human α S transgene expression by comparison (Teravskis et al., 2018). We previously showed that TgWT and TgA30P neurons exhibit presynaptic deficits as indicated by reductions in the probability of neurotransmitter release, but intact postsynaptic function and long-term potentiation (LTP) (Teravskis et al., 2018). Thus, we employed these models to directly test whether presynaptic deficits alone are sufficient to cause impairments in learning and memory. Spatial learning and memory analysis of 12-month-old TgWT, TgA30P, and nTg littermates using the BM paradigm shows that all groups of mice are able to learn and retain goal quadrant and escape box location (**Figure 2.2b-d**). Taken together, our findings show that α S-dependent presynaptic impairments alone are not sufficient to cause memory deficits in mouse models of α -synucleinopathy under our experimental conditions, and that memory deficits in TgA53T mice involves both pre and postsynaptic abnormalities.

To better define memory deficits in TgA53T mice, we assessed the progressive nature these deficits hippocampal-dependent spatial learning and memory via BM and whether endogenous tau was required for cognitive deficits in TgA53T mice. At 6 months of age, TgA53T mice exhibit a slight delay in acquisition compared to the nTg and mTau^{-/-} controls (**Figure 2.3b**), mitigated by extra training as they successfully learn the location of the escape hole in the goal quadrant in a timing similar to controls by training day 4 (**Figure 2.3b**). Consistent with previous data, 12-month-old TgA53T mice display severe impairments in the ability to learn the BM task, as they failed to display improved performance over consecutive training days (**Figure 2.3c**). Significantly, the loss of tau completely reverses the h α S^{A53T}-mediated learning deficit exhibited by TgA53T mice as TgA53T/mTau^{-/-} showed normal learning during training in the acquisition phase (**Figure 2.3c**).

In the ensuing probe trial, a putative measure for spatial learning and memory retention, all 6-month-old animals, including TgA53T, spent significantly more time in the goal quadrant compared to other quadrants in the maze (**Figure 2.3b and d, and Figure 2.4a and f**), indicating that TgA53T mice have normal long-term spatial memory at 6 months of age. By 12 months, TgA53T mice did not show evidence of spatial memory retention during the probe trial, consistent with impaired memory acquisition (**Figures 2.3c-d, and Figure 2.4b and f**). The memory deficits in the 12-month-old TgA53T mice is independent of motor deficits as the total distance traveled during the probe test was comparable between groups at both 6 and 12 months of age (**Figure 2.4c-d**).

Consistent with the normal learning and acquisition exhibited by TgA53T/mTau^{-/-} mice, loss of tau expression completely rescues TgA53T-associated memory deficits as 12-month-old TgA53T/mTau^{-/-} effectively learn and recall escape hole location within the goal quadrant. Collectively, these results demonstrate that tau expression is required for the progressive spatial learning and memory deficits in the TgA53T model.

TgA53T mice exhibit deficits in multiple memory modalities

While TgA53T mice demonstrate intact spatial learning and memory relative to nTg littermates at 6 months of age, hippocampal LTP deficits occur by 6 months in this model (Teravskis et al., 2018). Given that TgA53T mice may display mild BM learning deficits in early training days at 6 months of age, we hypothesized that TgA53T mice may exhibit more obvious tau-dependent deficits at earlier ages in other memory modalities involving hippocampal function. We first used contextual fear conditioning (CFC) to determine if TgA53T mice show deficits in this dual hippocampal and amygdala-dependent task (Bach et al., 1995; McKernan and Shinnick-Gallagher, 1997; Phillips and LeDoux, 1992). 3-month-old TgA53T mice show normal learning and memory comparable to the nTg littermates (**Figure 2.5b-d**). At 6 months of age, TgA53T mice respond similarly to foot

shocks during the context conditioning trial as compared to nTg, TgA53T/mTau^{-/-}, and mTau^{-/-} cohort counterparts (**Figure 2.5e**), indicating that these mice do not exhibit gross sensorimotor abnormalities that could confound these findings. TgA53T mice exhibit significantly less freezing when exposed to the “similar” context as compared to the other three strains tested (**Figure 2.5f**), indicating that CFC is impaired in 6-month-old TgA53T mice. All groups displayed reduced freezing tendencies in the “novel” setting, controlling for any sensorimotor differences (**Figure 2.5d and g**). Significantly, TgA53T/mTau^{-/-} animals showed equivalent CFC capacities compared to control mice (nTg and mTau^{-/-}), demonstrating that tau is required for mutant α S-dependent deficits in CFC.

Both the BM and CFC represent situations that subject mice to stressful environments to promote task performance. Thus, to reduce any confounds associated with the differential stress responses, we asked whether TgA53T mice have cognitive deficits in short-term memory using a spatial variant of the Y maze (YM), a less stressful assessment of spatial learning and memory (Conrad et al., 1996). Animals undergo an initial learning trial where the “novel” arm is blocked off followed by a “recognition” trial where all arms are open for exploration (**Figure 2.6a**). The recognition trial takes advantage of an animal’s propensity to explore novel objects and environments and tests their capacity to discriminate between novel and familiar environments (Antunes and Biala, 2012; Tagliabata et al., 2009). As such, measuring the amount of time spent in the novel (N) versus familiar (F) arms of the YM provides an assessment of short-term spatial learning and memory. 6-month-old nTg mice spent significantly more time in the N arm while littermate TgA53T mice spent equal amounts (chance) between N and F arms during the recognition trial (**Figure 2.6b and d**). The h α S^{A53T}-mediated reductions in spatial discrimination is suggestive of an impaired capacity for this hippocampal-dependent short-term spatial memory at 6 months of age. More importantly, the YM spatial discrimination

deficit was reversed in TgA53T/mTau^{-/-} mice, indicating that endogenous tau expression is required for hαS^{A53T}-associated impairments in both short- and long-term spatial learning and memory (**Figure 2.6b and d**). In TgA53T mice, Lewy-like amyloid intraneuronal accumulations of hαS are found primarily the dorsal midbrain, cerebellar nuclei, brainstem, and spinal cord, accompanied by astrogliosis but no neurodegeneration (Lee et al., 2002). However, these neuropathological changes are absent in cortical and limbic system structures, including the hippocampus and amygdala, of TgA53T mice brains (Lee et al., 2002) suggesting that hαS^{A53T}-mediated neuronal dysfunction can occur in the absence of aggregate formation. This observation is consistent with another hαS^{A53T}-expressing transgenic mouse line that develops motor and gait deficits but no αS-positive intracellular inclusions (Gispert et al., 2003).

Presymptomatic motor hyperactivity of TgA53T mice is tau-independent

Our results so far indicate that removing tau expression restores learning and memory deficits in TgA53T mice, particularly those associated with intact hippocampal function. However, TgA53T mice exhibit other presymptomatic abnormalities in advance of overt α-synucleinopathy-associated motor dysfunction presentation. In particular, TgA53T mice, but not TgWT and TgA30P, exhibit spontaneous locomotor hyperactivity associated with increased striatal D1 dopamine receptor sensitivity (Graham and Sidhu, 2010; Unger et al., 2006; Zhuang et al., 2001). Thus, we tested whether endogenous tau was required for hαS^{A53T}-dependent hyperactivity. As expected, TgA53T mice exhibit a progressive increase in locomotor hyperactivity as measured by distance traveled in the open field arena at 3, 6, and 12 months. This hyperactivity was tau-independent as TgA53T and TgA53T/mTau^{-/-} mice exhibit similar levels of hyperactivity that progress in a nearly-identical, age-dependent manner as well (**Figure 2.7b-c**). While tau is required for mutant

α S-mediated deficits in hippocampal function, we conclude that mutant α S-driven abnormalities associated with nigro-striatal circuitry appear tau-independent, suggesting a cell-type specificity of pathologic interactions between α S and tau.

Tau is required for A53T α S-linked impairments in excitatory and evoked neuronal activity

In our model for how mutant α S produces cognitive deficits, we propose that mutant α S causes tau-dependent postsynaptic deficits characterized by the loss of AMPAR and subsequent deficits in hippocampal glutamatergic signaling, LTP, and memory impairments (Teravskis et al., 2018). However, it is unknown if these pathways are directly interrelated. We therefore examined if endogenous tau expression was also required for α S-mediated synaptic deficits using acute hippocampal slice recordings (Teravskis et al., 2018). Further, to determine if these neurophysiological deficits in TgA53T neurons are progressive, we examined hippocampal slices from mice at 2-3 months of age, where animals have intact cognition, and 5-6 months of age, where mice first display impairments in CFC and YM. Consistent with our previous study, neurons from all mice show similar profiles in analysis of basal synaptic transmission at both age points, including analysis of input/output curves and paired-pulse facilitation (**Figure 2.8a-c**). Analysis of synaptic fatigue shows that neurons lacking tau, either mTau^{-/-} or TgA53T/mTau^{-/-}, exhibit variable attenuation of stimulus-dependent reductions in EPSC (excitatory postsynaptic current) amplitude, suggesting that tau may be involved in the regulation of high-frequency synaptic transmission under physiological conditions (**Figure 2.8f**). Neurons from 6-month-old TgA53T mice exhibit reduced synaptic strength as measured by comparing AMPAR currents to NMDA receptor (NMDAR) currents (AMPA/NMDA ratio) (**Figure 2.8g-h**). By contrast, neurons from 2-3-month-old TgA53T mice show a normal AMPA/NMDA ratio when compared to nTg littermate controls (**Figure 2.8g-h**), indicating that h α S^{A53T}-

mediated reductions in synaptic strength are progressive. Significantly, loss of tau expression in TgA53T/mTau^{-/-} reversed TgA53T-associated reductions in the AMPA/NMDA ratio (**Figure 2.8g-i**) providing the first *ex vivo* confirmation that tau mediates mutant α S-driven deficits in synaptic function, and that these deficits coincide with onset of cognitive dysfunction.

Overexpression of synuclein-family proteins including h α S^{WT}, h α S^{A53T}, and β -synuclein (β S), have been shown to reduce presynaptic vesicle release and neurotransmission (Nemani et al., 2010). However, h α S^{A53T} causes reductions in both spontaneous pre- and postsynaptic activities without detectable synapse loss (Teravskis et al., 2018). Thus, we examined if tau expression modulates h α S^{A53T} reductions in synaptic activity by analyzing spontaneous neurotransmitter release and synaptic activity via recording of mini excitatory postsynaptic currents (mEPSCs). mEPSC frequency, reflecting the probability of neurotransmitter release from presynaptic vesicles, is reduced in TgA53T neurons at both 2-3 months and 5-6 months of age, confirming that inhibition of neurotransmission is a direct, primary synaptic effect of α S expression (**Figure 2.9a and c-d**). More important, mEPSC amplitude, reflecting postsynaptic AMPAR function, were comparable between TgA53T and nTg neurons at 2-3 month of age, but show significant reductions in slices from 5-6-month-old animals (**Figure 2.9b-d**). These results, similar to memory deficits, indicate that postsynaptic deficits in TgA53T neurons are progressive with aging. Both mEPSC amplitude and frequency measured in neurons from TgA53T/mTau^{-/-} slices are not different from nTg and mTau^{-/-} controls, demonstrating that h α S^{A53T}-mediated pre and postsynaptic deficits require tau expression. The fact that mTau^{-/-} neurons were not different from nTg neurons in these measures demonstrates that the reversal of mutant α S-dependent effects in TgA53T/mTau^{-/-} neurons is not due to simple additive effects.

It is possible that the findings in acute hippocampal slices could involve tau-dependent alterations in circuit development or compensation. To account for this possibility, we used dissociated hippocampal neuronal cultures to test whether loss of tau can reverse the synaptic changes observed in TgA53T neurons (Teravskis et al., 2018). Neurophysiological analysis of primary cultures from TgA53T and TgA53T/mTau^{-/-} shows that loss of tau expression blocks the effects of mutant α S overexpression (**Figure 2.10**), implicating tau as a direct mediator of mutant α S-induced pre and postsynaptic changes.

Having established tau as a mediator of h α S^{A53T}-driven postsynaptic deficits in spontaneous neurotransmission, we next asked if tau contributes to A53T α S-mediated deficits in long-term plasticity by evaluating hippocampal LTP induction (Malinow and Malenka, 2002). Consistent with intact spatial learning and memory (**Figures 2.3-2.6**), LTP in TgA53T mice at 2-3 months is normal when compared to age-matched nTg littermates (**Figure 2.11a and c-d**). At 6 months, cognitively impaired TgA53T mice display tau-dependent memory deficits (**Figures 2.5-2.6**) and LTP deficits (**Figure 2.11b-c and e**). Taken together, our findings thus far demonstrate that tau is required for TgA53T-associated progressive impairments in synaptic activity and plasticity and spatial learning and memory. Moreover, our results show that presynaptic effects due to α S overexpression are not sufficient to cause memory deficits, suggesting that altered postsynaptic glutamatergic signaling is required for cognitive dysfunction in the TgA53T model.

Tau does not alter expression or oligomerization of α S in forebrains of TgA53T mice

Post-translational modifications of α S are believed to be integral to PD pathophysiology. Levels of aggregate-promoting C-terminally truncated α S (α S Δ C) are increased by familial PD-linked A30P and A53T missense mutations (Li et al., 2005). Further, phosphorylated α S at Serine 129 (pSer129) is an accepted marker of α S

pathology *in situ* (Fujiwara et al., 2002; Samuel et al., 2016). Thus, we asked if tau-dependent synaptic and memory deficits and cognitive decline in TgA53T mice were due to alterations in hippocampal and cortical α S expression, increased pathogenic α S modifications, α S oligomer accumulation, or abnormal tau accumulation. Immunoblot analysis demonstrates that total hippocampal and cortical tau levels were not different between nTg and TgA53T lysates, indicating that alterations in tau expression are not responsible for tau-dependent deficits in TgA53T mice and neurons. Conversely, the loss of tau also did not alter total α S levels in nTg or TgA53T mice (**Figure 12a-d, and Figure 13a and c**). In addition, both α S Δ C and pSer129 α S levels were unaffected by tau expression (**Figure 12a-b**). Finally, consistent with previous studies (Colla et al., 2012a; Lee et al., 2002; Li et al., 2005), very little levels of detergent-insoluble α S are present in hippocampal lysates and levels of detergent-insoluble α S are not altered by tau expression (**Figure 14**).

Analysis of total tau expression via Tau5 antibody indicates that TgA53T cortices and hippocampi express comparable tau levels to that of nTg brains (**Figure 12a-b, and Figure 13a and c**). To determine if the synaptic deficits observed in TgA53T neurons were associated with increased tau phosphorylation (Teravskis et al., 2018), expression of phosphorylated tau species as recognized by AT8, CP13, and PHF1 antibodies was determined via concurrent immunoblot analysis (Khan et al., 2018). These results demonstrate that TgA53T brains exhibit modest, but significantly increased levels of AT8, CP13, and PHF1-positive phosphorylated tau (**Figure 15**). As expected, no immunoreactivity to tau-related epitopes are observed in mTau^{-/-} animals. These results are consistent with our hypothesis that h α S^{A53T} drives synaptic deficits by phosphorylation-dependent mislocalization of tau into dendritic spines (Teravskis et al., 2018).

α S and tau also have the capacity to induce each other's aggregation and polymerization into fibrillar amyloid structures (Giasson et al., 2003) and α S oligomers can induce tau misfolding and downstream generation of oligomers (Castillo-Carranza et al., 2018; Gerson et al., 2018). Thus, we next investigated if the levels of soluble α S and/or tau oligomers could be correlated with synaptic and memory deficits in TgA53T mice. In all cortical and hippocampal lysates, no general amyloid structures (OC), soluble oligomeric species (A11), or tau oligomers (T22) (Lasagna-Reeves et al., 2012) were present at detectable levels (**Figure 12c-d, and Figure 16**). As expected, a panel of antibodies against conformational-specific and higher order assemblies of α S showed increased levels in TgA53T lysates. However, they did not reveal any changes as a function of tau expression: Syn33 (oligomers, dimers and higher molecular weight aggregates), F8H7 (oligomers from 70 kDa and above), and MJFR-14-6-4-2 (soluble oligomeric and fibrillar structures) (Lasagna-Reeves et al., 2012; Lindersson et al., 2004; Paleologou et al., 2009; Sengupta et al., 2015). While the mutant α S-selective effects on synaptic function and behavioral deficits suggest that pathological α S species are involved, our results indicate that the tau-dependent effects are not due to simple modulation of α S expression and α S oligomerization. Collectively, based on our results, we propose that the effect of tau may be occurring downstream or independent of the pathological conversion of α S.

Memory deficits in TgA53T mice occurs in the absence of synaptic degeneration

In vertebrates, α S is a part of the larger synuclein gene family that include homologs β - and γ -synuclein (George, 2002; Lavedan, 1998; Maroteaux and Scheller, 1991). β -synuclein (β S) can antagonize α S aggregation and toxicity (Hashimoto et al., 2001) and ameliorating endogenous β S enhances pathological α S phosphorylation and aggregation

(Fares et al., 2016). Consistent with previous findings (Thomas et al., 2011), expression of α S is inversely correlated with β S accumulation in the brain (**Figure 2.12e-f, and Figure 2.13b and d**). Interestingly, the reduction in cortical and hippocampal β S expression of TgA53T mice is not rescued by removal of endogenous tau (**Figure 2.12e-f, and Figure 2.13b and d**).

Other α S transgenic mouse models that exhibit memory deficits are associated with various levels of neurodegenerative changes, including cortical α S pathology and cortical and hippocampal synaptic loss (Hatami and Chesselet, 2015; Koprach et al., 2017). While cortical and hippocampal pathology are not robust features of the TgA53T model, we examined whether the tau-dependent memory deficits in the TgA53T model are due to loss of synapses by evaluating the protein abundance of presynaptic (synaptophysin) and postsynaptic (PSD95) proteins. Biochemically, there were no differences in the amounts of either synaptic proteins across the four genotypes assessed at 12 months of age (**Figure 2.12e-f, and Figure 2.13b and d**). We next extended our biochemical findings with immunostaining to probe for changes in hippocampal and synaptic structure. TgA53T hippocampi at 12 months show similar hippocampal structure as compared to age-matched nTg littermates via staining for neuronal nuclei (NeuN; **Figure 17a**), demonstrating that overt neuronal loss is not required for h α S^{A53T}-mediated cognitive decline. Furthermore, confocal images of somatodendritic (MAP2), presynaptic (synaptophysin), and postsynaptic (PSD95) structures qualitatively indicate intact synaptic structures in cognitively impaired 12 month TgA53T mice compared to nTg littermates (**Figure 17b**). Collectively, our results show that neither neuronal loss nor synaptic degradation are responsible for the α S-mediated cognitive dysfunction in TgA53T mice.

In light of an apparent intact synaptic integrity in TgA53T mice, increased human wild-type or mutant α S expression has been implicated in dysregulation of presynaptic function

by selectively reducing levels of synapsins (Larson et al., 2017; Nemani et al., 2010). Immunoblot analyses of cortical and hippocampal lysates confirm reduced levels of synapsin isoforms in TgA53T mice (**Figure 2.12e-f, and Figure 2.13b and d**). However, suppressed synapsin levels were not restored by loss of tau expression in TgA53T/mTau^{-/-} brains, indicating that tau functions in a distinct pathway or downstream of synapsin expression (**Figure 2.12e-f, and Figure 2.13b and d**). These results support our behavioral findings that α S-mediated presynaptic deficits alone are not sufficient for memory deficits.

Synaptic and memory deficits in TgA53T mice associated with tau-dependent alterations in postsynaptic glutamate receptors

The cellular and molecular mechanisms driving LTP are mediated by postsynaptic AMPAR and NMDAR (Bliss and Collingridge, 1993; Malinow and Malenka, 2002; Malinow et al., 2000). Tau missorting to dendritic spines is associated with memory loss and impairments in postsynaptic AMPAR signaling in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Alzheimer's disease (AD) (Hoover et al., 2010; Miller et al., 2014). We showed that h α S^{A53T}-mediated tau missorting to dendritic spines is associated with calcineurin-dependent AMPAR internalization (Teravskis et al., 2018). As the loss of hippocampal AMPARs likely contribute to deficits in LTP and memory, we sought to determine if reductions in AMPAR expression occurs in TgA53T mice. Immunoblot analysis displayed progressive reductions specific to hippocampal, but not cortical, AMPAR subunits (GluA1 and GluA2/3). Specifically, while AMPAR levels are not different between 3-month-old nTg and TgA53T mice, they are significantly reduced in TgA53T hippocampi at 6 and 12 months of age (**Figures 2.18**). These h α S^{A53T}-mediated deficits are tau-dependent as TgA53T/mTau^{-/-} hippocampi displayed levels of AMPAR expression similar to nTg and mTau^{-/-} controls at 12 months of age. Thus, loss of

hippocampal AMPAR levels correlate with age- and tau-dependent onset of synaptic and cognitive deficits. To determine the selectivity of AMPAR changes, we also examined NMDAR levels, receptors essential for LTP but not functionally altered by tau mislocalization to spines (Hoover et al., 2010). GluN1 and GluN2A expression remained unchanged in cognitively intact 3-month-old or impaired 6-month-old TgA53T mice compared to nTg controls (**Figure 2.19**). GluN1 expression was increased in 12-month-old TgA53T hippocampi and cortices, but, unlike AMPAR subunit expression changes, was neither changed by the status of tau expression (**Figure 2.18**). Collectively, these results parallel our neurophysiological findings and further our proposal that the TgA53T model is associated with the selective impairments in hippocampal postsynaptic AMPAR signaling in a tau-dependent manner while NMDARs are largely unaffected or slightly increased in multiple brain regions.

AD-associated amyloid- β ($A\beta$) oligomers have been shown to alter postsynaptic signaling through binding to cellular prion protein (PrP^C) that produces downstream Fyn activation and phosphorylation of the GluN2B subunit of NMDARs (Laurén et al., 2009; Um et al., 2012). It has recently been proposed that α S oligomers causes cognitive deficits through a mechanism involving activation of the cellular prion protein (PrP^C), Fyn, and GluN2B (Ferreira et al., 2017). We therefore examined whether PrP^C -Fyn-GluN2B signaling is altered in the TgA53T model. In contrast to this recent study (Ferreira et al., 2017), TgA53T mice did not show increased PrP^C expression, an established mechanism for mediating downstream increases in Fyn and GluN2B phosphorylation and activation (Ferreira et al., 2017; Um et al., 2012, 2013) (**Figure 2.20b-e**). We also determined that Fyn phosphorylation and activation was not increased in the TgA53T model (**Figure 2.21a-b**). The final output for pathological PrP^C and Fyn signaling is increased NMDAR signaling through phosphorylation of GluN2B by Fyn. Consistent with the absence of

increased PrP^C expression and Fyn activation, phosphorylation-inducing activation of GluN2B was not detected in TgA53T mice with either intact or impaired cognition (**Figure 2.21c-d**). Taken together, our results do not support involvement of PrP^C-Fyn-GluN2B signaling in the memory deficits observed in TgA53T mice. Our conclusion is also supported a recent study showing that PrP^C neither binds nor mediates the toxic effects of α S oligomers (La Vitola et al., 2019).

TgA53T mice exhibit progressive hippocampal inhibitory circuit remodeling

Disruption of neuronal networks due to chronic network hyperactivity has been hypothesized to be a potential mechanism contributing to cognitive decline in neurodegenerative diseases (Palop et al., 2006), particularly via increased spontaneous epileptiform activity and compensatory remodeling of networks (Chin et al., 2005; Palop et al., 2007; Sun et al., 2009). Thus, we asked if TgA53T mice exhibit evidence of hippocampal circuit remodeling typical of chronic network hyperactivity (Palop and Mucke, 2010; Palop et al., 2007): loss of c-Fos positive dentate granule cells, ectopic neuropeptide Y (NPY) expression in the mossy fiber pathway and molecular layer of dentate gyrus, and reduction in calbindin in the granule cells of dentate gyrus and stratum radiatum of CA1 (Morris et al., 2015; Palop et al., 2007; Roberson et al., 2011). At 3 months of age, cognitively intact TgA53T mice display c-Fos, NPY, and calbindin staining indistinguishable from nTg mice (**Figure 2.22b-d, and Figure 2.23a and c-e**). These results indicate that when mice display normal postsynaptic function and memory, hippocampal circuits have not been exposed to chronic network hyperactivity. At 6 months, two distinct populations of TgA53T are observed: one displaying circuit remodeling consistent with chronic network hyperactivity and another “intermediate” group without circuit remodeling (**Figure 2.22b-d, and Figure 2.23b-h**). By 12 months of age, all TgA53T mice show prominent network changes indicative of chronic epileptic activity

(**Figure 2.22**). Importantly, these age-dependent, α S-mediated alterations in hippocampal circuits are absent in the 12-month-old TgA53T/mTau^{-/-} mice. These results indicate that hippocampal network remodeling in the TgA53T model starts between 3 and 6 months of age, likely as a compensatory response to network hyperactivity. The variability observed in 6-month-old TgA53T mice is consistent with animals undergoing a transition period. Further, the TgA53T-linked inhibitory hippocampal circuit remodeling is absent in TgA53T/mTau^{-/-} animals, indicating that h α S^{A53T}-associated network hyperactivity, along with progressive synaptic and memory deficits, require endogenous tau expression.

Tau is a partial mediator of exogenous, fibrillar α S-induced disease onset in TgA53T mice

In a human mutant amyloid-precursor protein (hAPP) mouse model of AD, tau is required for A β -induced lethality (Roberson et al., 2007). Interestingly, dementia in PD is associated with increased mortality risk (Emre et al., 2007; Levy et al., 2002; Svenningsson et al., 2012; Willis et al., 2012). Bridging these two observations, we therefore next asked if tau was required for h α S^{A53T}-induced premature morbidity and mortality in an accelerated model of PD that employed exogenous fibrillar α S species (Volpicelli-Daley et al., 2014). Misfolded fibrillar forms of α S, are hypothesized to represent a pathogenic and transmissible species of the protein. Peripheral injection of fibrils into transgenic mouse models of synucleinopathy has been demonstrated to induce robust α S pathology in the CNS, progressive motor impairments, and premature lethality on the order of weeks post-injection, representing an appealing model for studying the mechanisms underlying α S-induced disease pathogenesis (Ayers et al., 2017; Sacino et al., 2014). To assess the role of tau in mediating these pathological changes, we generated human wild-type α S pre-formed fibrils (PFFs) in line with accepted protocols

(Volpicelli-Daley et al., 2014). Following this, 3-month-old mice received bilateral intramuscular injections of PFFs into the *biceps femoris muscle*. At this age, TgA53T animals in our colony do not display gross neurological and locomotor impairments, consistent with their original characterization (Lee et al., 2002). Post-injections, animals were monitored for two pre-defined phenotypic end points that would occur in sequential order: 1) disease onset and 2) end stage. PFF-induced disease-onset is characterized as an animal displaying hindlimb clasp and gait abnormalities (“wobbling”) when moving around in their home cage or on a level surface (**Figure 2.24d and f-g**). Following disease onset, the second end point of interest, termed end stage, is characterized as animals being moribund, no longer able to locomote, and displays cachexia due to inability to eat and drink (**Figure 2.24e-g**). The Kaplan-Meier curve analysis demonstrates that tau amelioration in TgA53T mice mildly, although significantly, slows the rate of PFF-induced disease onset and lethality in TgA53T animals (**Figure 2.24d-e**). Another component to the PFF model is disease progression, defined as the time between end point 1 (disease onset) and end point 2 (end stage). When assessing disease progression, there was no significant between TgA53T animals with or without endogenous mouse tau expression (**Figure 2.43f-g**). As such, it is possible that tau may be responsible in partially-mediating PFF-induced disease onset, explaining the subtle delay in TgA53T/mTau^{-/-}, that persists through end stage. However, it is critical to note that no PFF-injected nTg nor mTau^{-/-} mice became symptomatic or reached end point 1 during the time frame mice were monitored, during which all PFF-injected TgA53T and TgA53T/mTau^{-/-} reached both end points (**Fig 2.24d-e**). By that metric, it is evident that tau is not a major contributor to PFF-induced disease onset and lethality in mice expressing hαS^{A53T}, and it is likely that pathogenesis in this model may be occurring through pathways and mechanisms independent of tau.

Discussion

Dementia associated with α -synucleinopathy, particularly PDD and DLB, is one of the leading sources of global disability from neurological disorders (Dorsey et al., 2018; Jellinger and Korczyn, 2018). Currently, there are no effective therapies for managing PDD and DLB. Thus, understanding the cellular and molecular mechanisms of dementia in PDD and DLB will facilitate the development of new treatments. Previously, we proposed a model where $h\alpha S^{A53T}$ induces tau mislocalization to dendritic spines, leading to postsynaptic deficits in AMPAR signaling (Teravskis et al., 2018). However, it was not known if these tau-related changes are directly causative for deficits in synaptic plasticity and learning and memory in the TgA53T mouse model. In our present study we provide the first *in vivo* evaluation of the mechanistic relationships between αS abnormalities, tau expression, synaptic function, and memory. Our data shows that removal of endogenous tau expression reverses a range of synaptic and memory impairments in TgA53T mice. Further our results indicate that the effect of tau may be downstream or independent of potentially pathogenic αS oligomeric or fibrillar species. Because TgA53T-associated deficits in synaptic function and learning and memory are not connected with hippocampal neurodegeneration, it is likely that tau regulates $h\alpha S^{A53T}$ -alterations in synaptic physiology by perturbing neuronal function. In particular, our results indicate that tau-dependent loss of AMPAR function is an early event that is tightly correlated with LTP deficits, evidence of hippocampal network remodeling suggestive of chronic hyperactivity, and cognitive dysfunction in the TgA53T model.

A convergence of αS , tau, and $A\beta$ pathologies has been observed in PDD and DLB patients with increased cognitive impairment, suggesting a mechanism for neuronal dysfunction in α -synucleinopathies involving more than αS pathology (Colom-Cadena et al., 2013; Irwin et al., 2013). Significantly, dementia, cortical and hippocampal αS pathology, and tau pathology are often noted features of PD patients with A53T mutation

in α S (Bougea et al., 2017; Duda et al., 2002; Golbe et al., 1990; Markopoulou et al., 2008; Spira et al., 2001). In this study, we expand on the interaction between α S and tau by showing that physiological tau expression independent contributes to neurophysiological and memory deficits in TgA53T mice. While we were not able to document accumulation of pathological tau oligomers, others have shown that pathological accumulation of tau oligomers in another TgA53T mouse model (Gerson et al., 2018), suggesting that tau may act downstream of α S pathology. Our results also add to the role of endogenous tau expression as a mediator of neuropathological phenotypes in AD models (Ittner et al., 2010; Roberson et al., 2007, 2011; Vossel et al., 2010). The requirement of tau for memory deficits and the immunohistochemical markers of chronic epileptiform activity in the TgA53T model is reminiscent of studies in the hAPP-J20 transgenic mouse model of AD, where loss of tau expression reversed memory deficits and epileptic activity (Palop et al., 2007; Roberson et al., 2007, 2011). In hAPP-J20 mice, chronic epileptiform activity is mechanistically linked to cognitive deficits. Thus, it is likely that network hyperactivity, as indicated by age-dependent remodeling of inhibitory networks in TgA53T mice, also contributes to memory deficits in the TgA53T mouse model. Significantly, another model of α S-dependent memory deficits (Thy1-h α S: line 61) (Hatami and Chesselet, 2015) also shows increased epileptic activity, which was partially dependent on endogenous mouse tau expression (Morris et al., 2015). However, it is unknown if tau is required for memory deficits in the Thy1-h α S model or in other models of α S-dependent synaptic and memory deficits. The pathologic link between epileptic activity and memory deficits may be relevant for understanding DLB as both AD and DLB are associated with higher incidences of seizures and signs of seizure-like network hyperactivity (Beagle et al., 2017; Martinez-Losa et al., 2018; Minkeviciene et al., 2009; Morris et al., 2015; Palop et al., 2007; Roberson et al., 2011; Sun et al., 2009; Verret et al., 2012).

The progressive cognitive decline in TgA53T mice first observed at 6 months of age appears to correlate primarily with the loss of AMPAR expression leading to deficits in postsynaptic activity and LTP. Interestingly, TgA53T mice at this age point do not consistently display the molecular signature for chronic network hyperactivity of reductions in dentate granule cell c-Fos expression, increased ectopic NPY expression, and calbindin depletion in dentate granule cells, suggesting that the synaptic and memory deficits in the TgA53T model seem to precede inhibitory network remodeling. However, because network remodeling is a likely response to chronic and aberrant network hyperactivity (Palop and Mucke, 2010, 2016; Palop et al., 2006), we propose that the onset of this epileptiform activity is coincident with other synaptic deficits. It will be of significant future interest to determine the causal relationship between synaptic deficits, epileptic activity, and memory deficits as it has been studied in the hAPP-J20 model (Sanchez et al., 2012).

While several transgenic A β and α S mouse models share a final common outcome of memory loss through impairments in synaptic and neural network function, the mechanistic details appear to differ. One hypothesis for A β -mediated cognitive decline involves postsynaptic dysfunction via activation of the PrP^C-Fyn pathway, leading to phosphorylation and activation of the NMDAR subunit GluN2B, which, in turn, culminates in excitotoxicity and spine loss (Gimbel et al., 2010; Laurén et al., 2009; Roberson et al., 2011; Um et al., 2012). This pathway has also been recently implicated in memory loss associated with α -synucleinopathies (Ferreira et al., 2017). However, in our studies, we did not observe PrP^C-Fyn-GluN2B activation in TgA53T mice, indicating that h α S^{A53T} produces progressive postsynaptic and cognitive deficits through a mechanism independent of this pathway. Interestingly, in the hAPP-J20 model of AD, which, like TgA53T model of α -synucleinopathy, requires tau as a mediator of synaptic dysfunction and memory loss (Roberson et al., 2007), PrP^C and Fyn activation appears to be

mechanistically disconnected as tau-dependent cognitive decline in hAPP-J20 mice is unaffected by PrP^C ablation (Cissé et al., 2011). Furthermore, this view is consistent with a recent report (La Vitola et al., 2019) showing that, in contrast to the findings of Ferreira et al. (2017), PrP^C does not mediate the detrimental effects of α S oligomers. Our results suggest that synaptic and memory deficits in the TgA53T model appear to be independent of alterations in NMDAR but associated with decreased AMPAR subunit expression. Finally, consistent with the lack of Fyn-GluN2B activation, we do not observe a significant loss of postsynaptic structures in aged TgA53T mice.

Pathological α S can spread throughout the central nervous system, including cortical and hippocampal neurons, in a hierarchal progression that appears to follow functionally connected regions (Braak and Del Tredici, 2008; Braak et al., 2003), raising the possibility that certain neurodegenerative processes in PD may be produced by abnormal network activity. Supporting this mechanistic view, studies link PDD and DLB with LNs in the hippocampal CA2/3 region (Adamowicz et al., 2017; Hall et al., 2014). Here, we provide evidence where pathogenic α S causes reduced synaptic activity at specific excitatory synapses in the hippocampus, leading to deficits in AMPAR signaling and LTP. However, we also show that pathogenic h α S^{A53T} concurrently elicits aberrant excitatory activity as indicated by prominent compensatory remodeling of the inhibitory hippocampal circuits. One hypothesis to reconcile these seemingly contradictory observations is that depression of excitatory synaptic activity may be caused by a compensatory synaptic scaling mechanism reminiscent of the pro-excitatory effects of A β . For example, while acute oligomeric A β or α S application has been shown to transiently increase AMPAR and NMDAR on the neuronal surface (Ferreira et al., 2017; Sanchez-Mejia et al., 2008), prolonged A β oligomer exposure decreases AMPAR and NMDAR and suppresses synaptic plasticity (Hsieh et al., 2006; Shankar et al., 2007).

In our study, deficits in postsynaptic function, LTP, and memory are selectively associated with the expression of $h\alpha S^{A53T}$. Since increased expression of α - or β -synucleins lead to presynaptic deficits (Nemani et al., 2010; Teravskis et al., 2018), we conclude that α S-associated presynaptic deficit is not sufficient to cause LTP and cognitive decline. However, memory deficits have been documented in other transgenic mouse models expressing $h\alpha S^{WT}$ or $h\alpha S^{A30P}$ under the Thy-1 promoter (Hatami and Chesselet, 2015). Thus, under appropriate circumstances, it is clear that multiple $h\alpha S$ variants can cause cognitive dysfunction. Currently, it is unknown if the postsynaptic deficits observed in our TgA53T animals are associated with memory deficits in these other TgWT or TgA30P mouse models. However, increased epileptic activity has been documented in the TgWT Thy1- $h\alpha S$ model (line 61) (Morris et al., 2015), suggesting possible common mechanisms underlying α S-dependent synaptic and network deficits. We propose that a number of factors, including variations in transgene expression, background of mouse strains, housing conditions, and testing conditions and parameters could all contribute to the accumulation of toxic α S species that cause of memory deficits in TgWT and TgA30P models. It will be of significance in the future to determine if tau is required for memory deficits in multiple transgenic mouse models of α -synucleinopathy.

Although synaptic loss is the major correlate for memory loss in AD patients (Scheff et al., 2006; Selkoe, 2002; Terry et al., 1991; de Wilde et al., 2016), clinical and pathological studies suggest that cognitive deficits in PDD/DLB occurs in the absence of large scale loss of hippocampal neurons (Bougea et al., 2017; Hely et al., 2008; Irwin et al., 2012; Kantarci et al., 2016). Further, while the amount of tau pathology correlates with the severity of dementia (Coughlin et al., 2018), tau pathology per se is not as strongly linked to the incidence of dementia in PDD and DLB as α S or $A\beta$ pathology (Hall et al., 2014; Irwin et al., 2012; Jellinger and Korczyn, 2018; Kalaitzakis et al., 2009). Significantly, the

MAPT H1 haplotype increases risk for PDD and DLB (Goris et al., 2007; Orme et al., 2018). Since the *MAPT* H1 haplotype is thought to increase tau expression (Myers et al., 2007), it is possible that basal tau expression, independent of tau pathology, contributes to α S-dependent memory impairments in humans.

Prior to our current study, it was previously unknown how pathologic and genetic observations associated with PDD and DLB are related to the development of deficits in synaptic plasticity that underlie cognitive dysfunction. We provide evidence that the TgA53T model exhibits progressive, tau-dependent, memory loss associated with postsynaptic AMPAR dysfunction in the absence of overt synaptic loss and hippocampal neurodegeneration. More importantly, our results suggest that endogenous tau is required for the specific synaptic and memory deficits observed in TgA53T mice. Additionally, we propose that h α S^{A53T} causes tau-dependent synaptic alterations that lead to increased network hyperactivity that contribute to and exacerbate memory deficits (**Figure 2.25**). Our results, combined with previous studies in AD models, suggest that network hyperexcitability represents a novel therapeutic target for treating this driver of cognitive dysfunction in α -synucleinopathies. Collectively, this study introduces a mechanism that identifies key proteins and mechanisms that could mediate the neuronal dysfunction underlying memory deficits in PDD and DLB. Ultimately, building a more complete picture of how pathologic α S produces memory deficits is essential for developing novel therapeutic strategies for PDD and DLB.

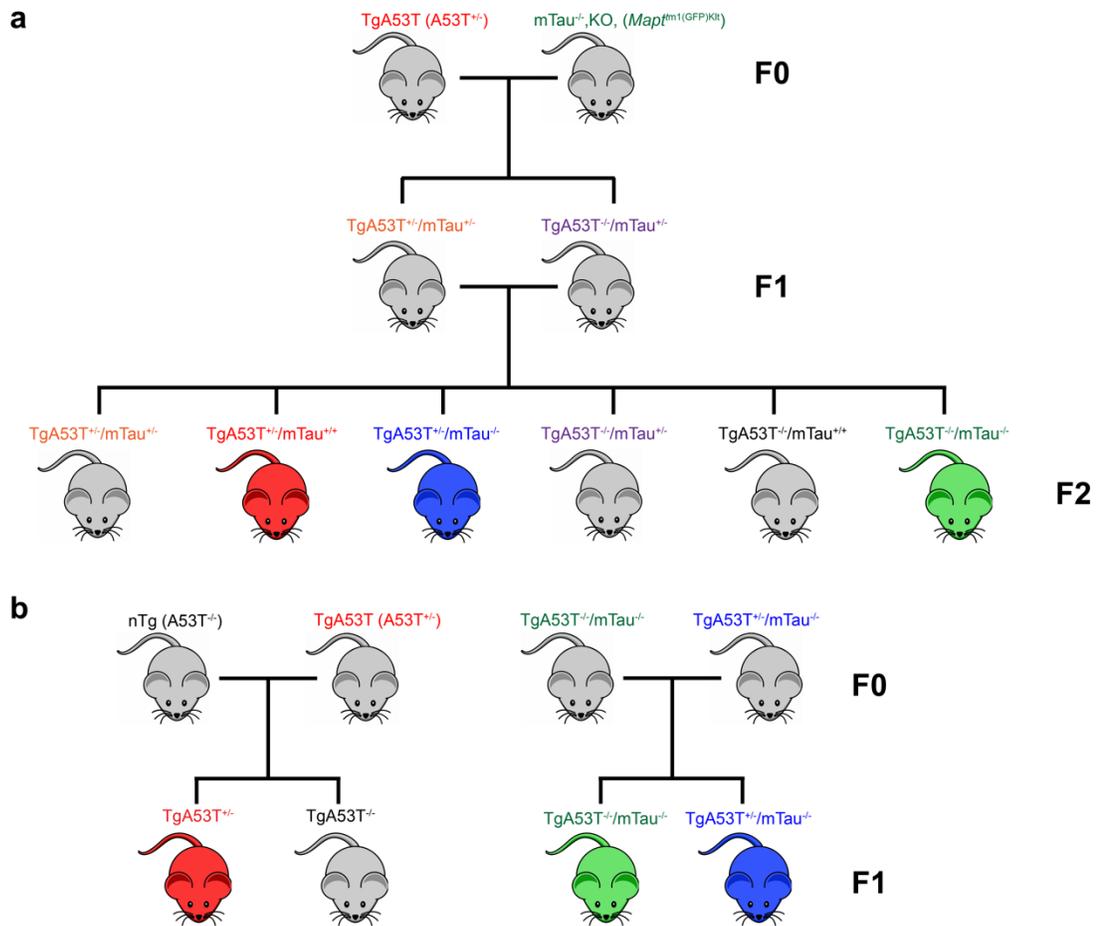


Figure 2.1 – Breeding scheme to generate experimental animals and cohorts. a. Initial breeding diagram from parents (F0) to generation of F2 progeny that provides mice with appropriate genotypes to use for downstream breeding for generation of experimental cohorts. **b.** F0 parents and breeding approach to generation of offspring that creates our four genotypes (nTg, TgA53T, TgA53T/mTau^{-/-}, mTau^{-/-}) of interest without generating additional animals (mTau^{+/-} heterozygotes) that would not be experimentally useful.

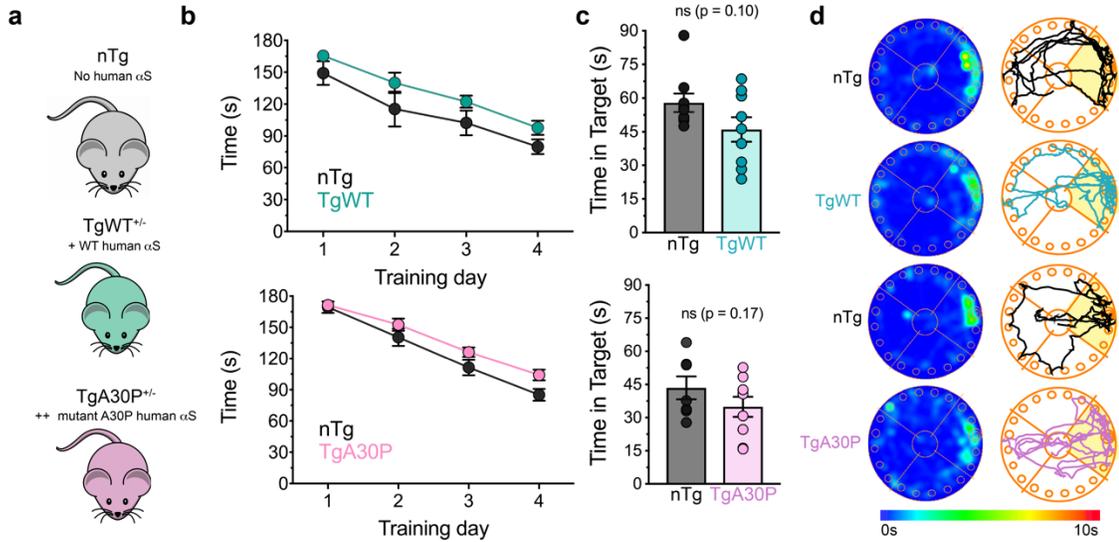


Figure 2.2 – Aged TgWT and TgA30P mice do not display deficits in long-term spatial learning and memory via Barnes Maze. **a.** Depiction of animals used: wild-type animals (nTg) do not express human α S. TgWT animals are heterozygous for a transgene that expresses wild-type human α S. TgA30P animals are heterozygous a transgene that expressed human mutant A30P α S. **b.** Barnes maze (BM) training trials for 12-month-old nTg, TgA30P, and TgWT animals demonstrating duration of training trials per group during each of the four training days. TgWT training: two-way repeated measures ANOVA with Geisser-Greenhouse correction and Sidak's posthoc analysis revealed a significant effect of training ($F_{(2,545,40.72)} = 36.09$, $p < 0.0001$), no effect of $h\alpha S^{WT}$ genotype ($F_{(1,16)} = 3.249$, $p = 0.0903$), and no significant training day* $h\alpha S^{WT}$ interaction ($F_{(3,48)} = 0.1449$, $p = 0.9325$). TgA30P training: two-way repeated measures ANOVA with Geisser-Greenhouse correction and Sidak's posthoc analysis revealed a significant effect of training ($F_{(1,838,29.40)} = 143.9$, $p < 0.0001$), no effect of $h\alpha S^{A30P}$ genotype ($F_{(1,16)} = 2.806$, $p = 0.1113$), and no significant training day* $h\alpha S^{A30P}$ interaction ($F_{(3,48)} = 1.645$, $p = 0.1915$). **c.** Time (seconds) spent occupying the target (goal) quadrant of the Barnes maze during the probe trial. Unpaired t test with Welch's correction. TgWT: $t = 1.472$, $df = 14.91$; TgA30P: $t = 1.439$, $df = 15$. TgWT: $N = 9$ animals/genotype. TgA30P: $n_{nTg} = 7$; $n_{TgA30P} = 9$. **d.** Left column: probe test BM occupancy heat maps obtained by averaging the location of all animals in each genotype and cohort. Right column: representative individual animal traces tracking movement during the BM probe test. BM orientation shown in Fig 1c, yellow shading indicates goal quadrant. The findings here demonstrate that postsynaptic deficits, as observed in TgA53T mice but not in TgWT or TgA30P, are required for cognitive decline by 12 months of age as TgWT and TgA30P do not display such deficits at this time point. ns: not significant. Error bars represent mean \pm standard error of the mean (S.E.M). Contributions: CG.

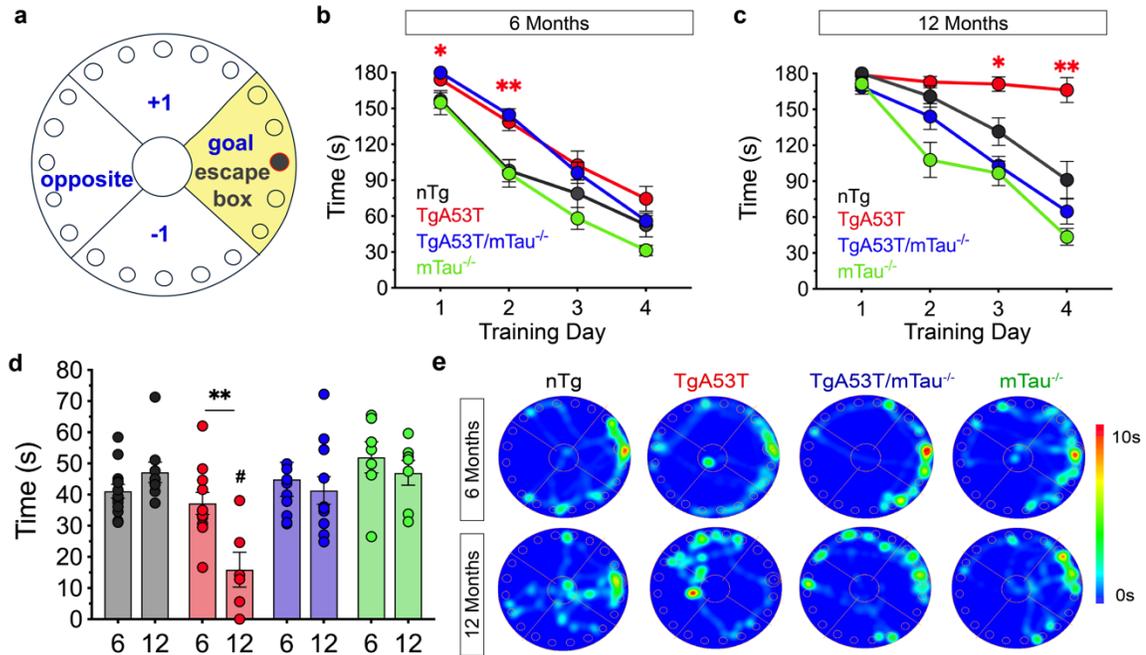


Figure 2.3 – Human mutant A53T α S driven deficits in spatial learning and memory are progressive and tau-dependent. **a.** BM diagram for testing and probe trial, with yellow shading indicating goal quadrant and dark grey showing escape box location in that quadrant. **b and c.** Average duration of training trials per group for each of the 4 training days during the Barnes Maze (BM) when tested at either 6 (**b**) or 12 months of age (**c**) (6M or 12M, respectively). **b.** 6M training: three-way repeated measures ANOVA with Geisser-Greenhouse correction and Tukey's posthoc analysis revealed a significant effect of training day ($F_{(2,448,105.3)} = 190.7, p < 0.0001$), significant effect of $h\alpha S^{A53T}$ genotype ($F_{(1,43)} = 18.63, p < 0.0001$), no significant effect of $mTau^{-/-}$ genotype ($F_{(1,43)} = 1.135, p = 0.2926$), and no significant training day* $h\alpha S^{A53T}$ * $mTau^{-/-}$ interaction ($F_{(3,129)} = 0.1160, p = 0.9506$). One-way ANOVA with Tukey's posthoc analysis to compare within 6M training days (TD): TD 1: $F_{(3,43)} = 5.856, p = 0.0019$; TD 2: $F_{(3,43)} = 9.277, p < 0.0001$; TD 3: $F_{(3,43)} = 2.466, p = 0.0768$; TD 4: $F_{(3,43)} = 2.189, p = 0.2330$. **c.** 12M training: three-way repeated measures ANOVA with Geisser-Greenhouse correction and Tukey's posthoc analysis revealed a significant effects of training day ($F_{(2,192,63.56)} = 62.15, p < 0.0001$), $h\alpha S^{A53T}$ genotype ($F_{(1,29)} = 13.78, p = 0.0009$), and $mTau^{-/-}$ genotype ($F_{(1,29)} = 49.05, p < 0.0001$), and no significant training day* $h\alpha S^{A53T}$ * $mTau^{-/-}$ interaction ($F_{(3,87)} = 3.734, p = 0.0141$). One-way ANOVA with Tukey's posthoc analysis to compare within 12M training days (TD): TD 1: $F_{(3,29)} = 1.407, p = 0.2607$; TD 2: $F_{(3,29)} = 5.664, p = 0.0035$; TD 3: $F_{(3,29)} = 10.64, p < 0.0001$; TD 4: $F_{(3,29)} = 15.82, p < 0.0001$. **d.** Time spent in goal quadrant during probe trial for 6M or 12M mice. To compare within genotype between 6M and 12M: unpaired t test with Welch's correction; TgA53T: $t = 3.369$ and $df = 15$ (** $p = 0.0042$). To compare between genotypes at 6M or 12M: one-way ANOVA with Tukey's posthoc analysis. 12M group probe test: $F_{(3,29)} = 9.216$ (# $p = 0.0002$). 6M: $n_{nTg} = 13$; $n_{TgA53T} = 11$; $n_{TgA53T/mTau^{-/-}} = 10$; $n_{mTau^{-/-}} = 8$. 12M: $n_{nTg} = 9$; $n_{TgA53T} = 7$; $n_{TgA53T/mTau^{-/-}} = 11$; $n_{mTau^{-/-}} = 8$. **e.** Probe test BM occupancy heat maps during probe test obtained by averaging the location of all animals in each genotype and cohort. Orientation of BM is shown in (**a**). In all figures: 1) the color code is: nTg (black), TgA53T (red), TgA53T/ $mTau^{-/-}$ (blue), and $mTau^{-/-}$ (green); 2) the data are expressed as mean \pm standard error of the mean (S.E.M.); and 3) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, unless stated otherwise. Contributions: MAB.

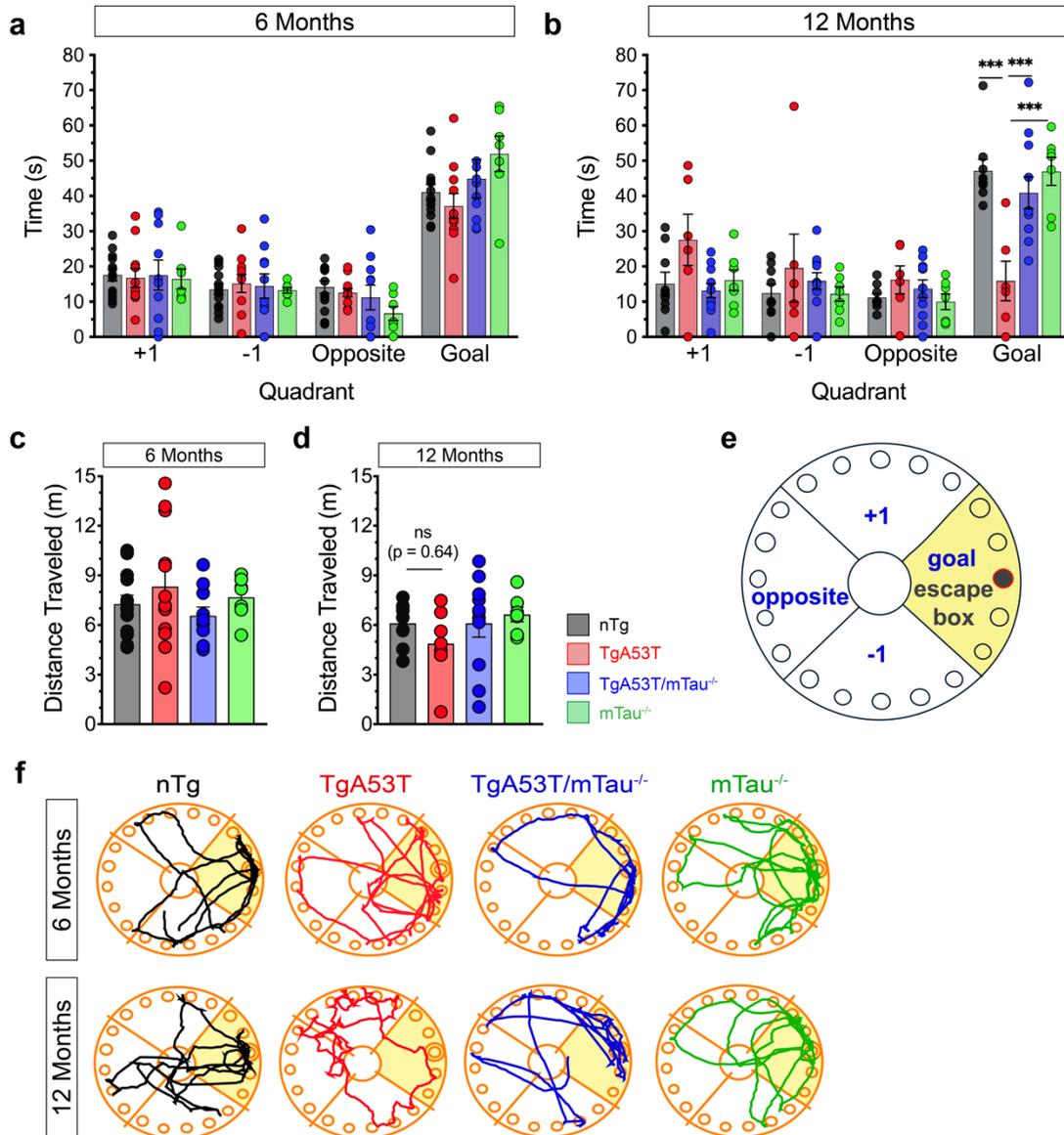


Figure 2.4 – Barnes Maze quadrant analysis demonstrating progressive deficits in long-term spatial learning and memory in TgA53T mice in absence of locomotor neurological deficits. a and b. Analysis of time animals spent in each quadrant of the Barnes Maze (BM) during the probe test of the BM at 6 months (6M) (**a**) and 12 months (12M) (**b**). 12M BM goal quadrant: $F_{(3,29)} = 10.47$, $p < 0.0001$, one-way ANOVA with Tukey's posthoc analysis. **c and d.** Total distance traveled on the BM by animals during the probe test at 6M (**c**) and 12M (**d**). 12M: $F_{(3,31)} = 0.9377$, $p = 0.4347$ by one-way ANOVA with Tukey's posthoc analysis. 6M: $n_{nTg} = 13$; $n_{TgA53T} = 11$; $n_{TgA53T/mTau^{-/-}} = 10$; $n_{mTau^{-/-}} = 8$. 12M: $n_{nTg} = 9$; $n_{TgA53T} = 7$; $n_{TgA53T/mTau^{-/-}} = 11$; $n_{mTau^{-/-}} = 8$. **e.** BM diagram for testing and probe trial, with yellow shading indicating goal quadrant and dark grey showing escape box location in that quadrant. **f.** Representative individual animal traces tracking movement during the BM probe test. Yellow shading indicates goal quadrant. These results demonstrate that TgA53T mice have age-dependent deficits in spatial learning and memory that are dependent on endogenous mouse tau expression and precede onset of locomotor abnormalities. One-way ANOVA: *** $p < 0.001$. ns: not significant. Error bars represent mean \pm S.E.M. Contributions: MAB.

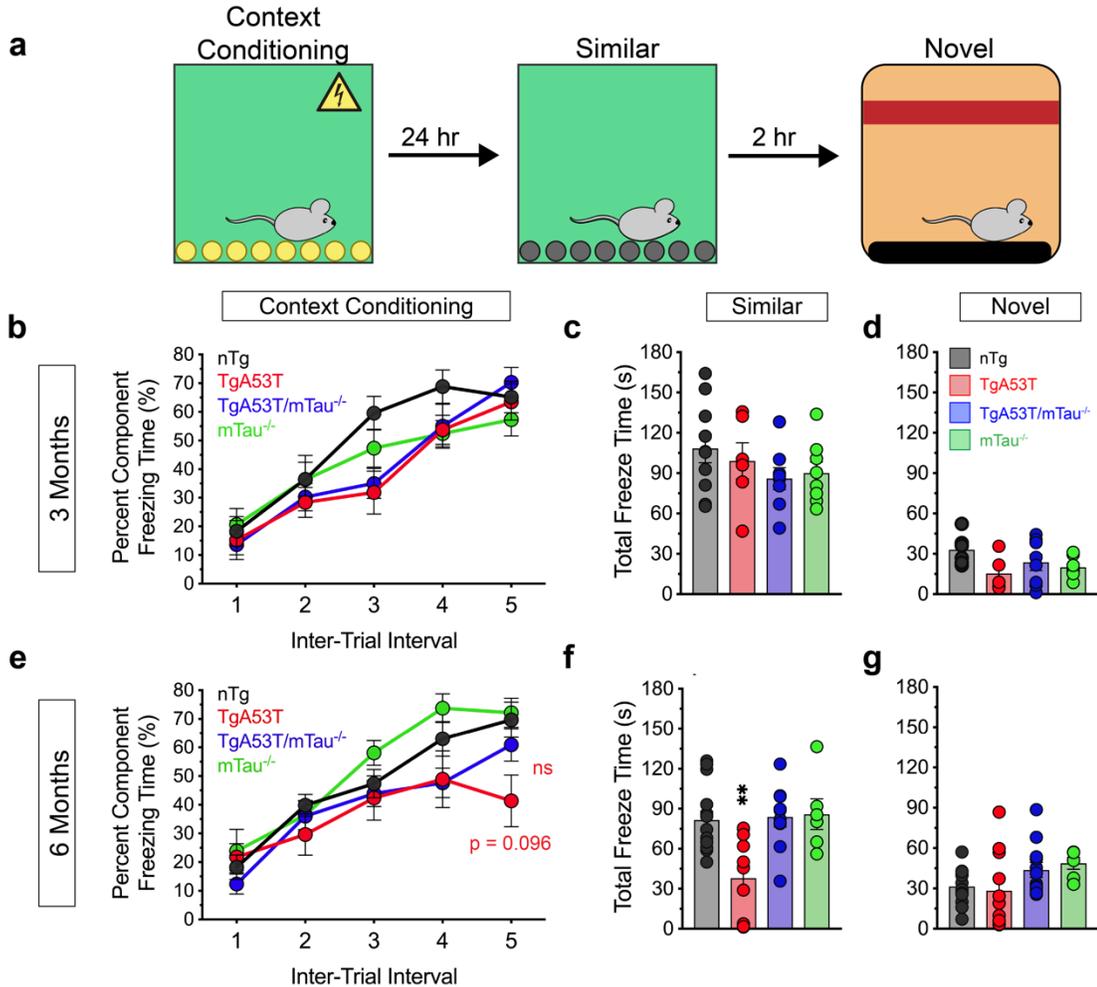


Figure 2.5 – Mutant synuclein impairs hippocampal contributions to contextual fear conditioning in both age and tau-dependent manners. **a.** Diagram of contextual fear conditioning (CFC) testing paradigm. CFC results in mice at 3 months (3M) (**b-d**) and 6 months (6M) of age (**e-g**). 3M conditioning trial (**b**): three-way repeated measures ANOVA with Geisser-Greenhouse correction and Tukey's posthoc analysis revealed a significant effect of inter-trial interval ($F_{(3,200,89.59)} = 67.91$, $p < 0.0001$), no significant effect of $h\alpha S^{A53T}$ genotype ($F_{(1,28)} = 2.123$, $p = 0.1562$), no significant effect of $mTau^{-/-}$ genotype ($F_{(1,28)} = 0.2536$, $p = 0.6185$), and no significant inter-trial interval* $h\alpha S^{A53T}$ * $mTau^{-/-}$ interaction ($F_{(4,112)} = 1.075$, $p = 0.3723$). 6M conditioning trial (**e**): three-way repeated measures ANOVA with Geisser-Greenhouse correction and Tukey's posthoc analysis revealed a significant effect of inter-trial interval ($F_{(3,175,120.6)} = 52.72$, $p < 0.0001$), a significant effect of $h\alpha S^{A53T}$ genotype ($F_{(1,38)} = 5.429$, $p = 0.0252$), no significant effect of $mTau^{-/-}$ genotype ($F_{(1,38)} = 0.7273$, $p = 0.3991$), and no significant inter-trial interval* $h\alpha S^{A53T}$ * $mTau^{-/-}$ interaction ($F_{(4,152)} = 2.257$, $p = 0.0655$). One-way ANOVA with Tukey's posthoc analysis was used to compare within 3M and 6M inter-trial intervals. 6M inter-trial interval 5: $F_{(3,38)} = 2.779$, $p = 0.0782$. Percent of time spent freezing during conditioning trials (**b and c**) show similar responses in all genotypes. When mice are exposed to the Similar environment, all genotypes show similar freezing responses at 3M (**c**) while 6M TgA53T mice freeze less than controls (**f**) (one-way ANOVA with Tukey's posthoc analysis: $F_{(3,37)} = 7.538$, $p = 0.0005$), indicating defective CFC. In contrast, 6M TgA53T/ $mTau^{-/-}$ mice show normal memory (**f**). Responses to the Novel environment are not different between groups at both 3M (**d**) and 6M (**e**). 3M: $n_{nTg} = 8$; $n_{TgA53T} = 7$; $n_{TgA53T/mTau^{-/-}} = 8$; $n_{mTau^{-/-}} = 9$. 6M: $n_{nTg} = 13$; $n_{TgA53T} = 11$; $n_{TgA53T/mTau^{-/-}} = 10$; $n_{mTau^{-/-}} = 8$. One- and three-way ANOVA: ** $p < 0.01$. ns: not significant. Error bars represent mean \pm S.E.M.

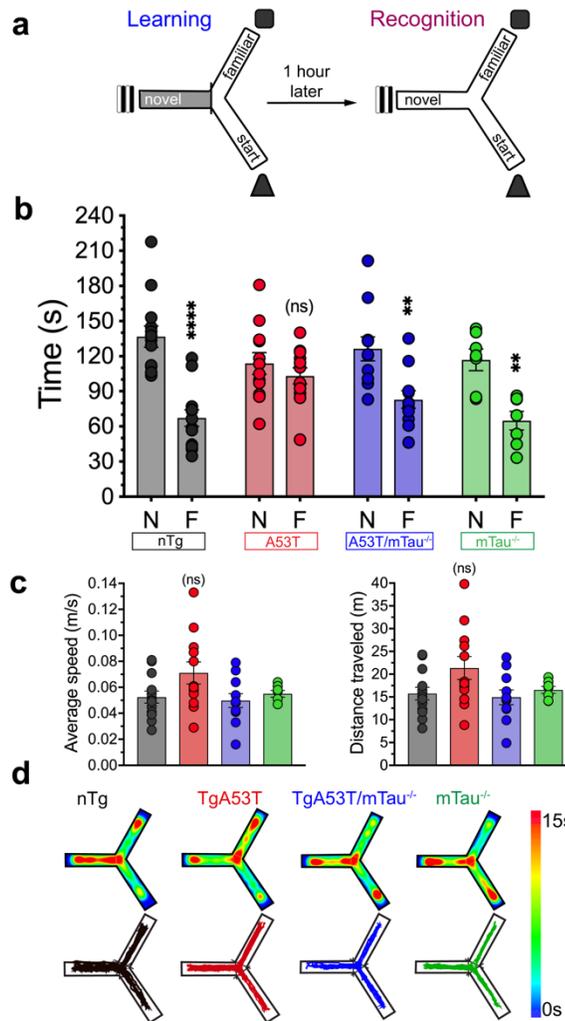


Figure 2.6 – TgA53T mice show impaired short-term spatial learning and memory in a tau-dependent manner. **a.** Diagram depicting the Y maze (YM) orientation and experimental testing paradigm. YM testing was divided into two discrete phases: first, a Learning trial (Novel arm blocked off), followed by a Recognition test (all arms open, including Novel). Walls at the end of each arm were marked by different patterns: start with triangles, familiar with square checkerboard, and novel with stripes. **b.** Time spent in novel (N) and familiar (F) arms of the Y maze during the 300-second-long recognition trial. Unpaired t test with Welch's correction was used compare time animals spent in N versus F arms during recognition trial within genotype. nTg: $t = 5.967$, $df = 22$, $p < 0.0001$; TgA53T: $t = 0.9192$, $df = 20.40$, $p = 0.3687$; TgA53T/mTau^{-/-}: $t = 3.425$, $df = 18.34$, $p = 0.0030$; mTau^{-/-}: $t = 4.291$, $df = 11$, $p = 0.0011$. **c.** Left panel: average speed (meters/second) of mice during the recognition trial: $F_{(3,39)} = 2.609$, $p = 0.0652$, one-way ANOVA with Tukey's posthoc analysis. Right panel: total distance traveled (meters) by animals during the recognition trial. $F_{(3,39)} = 2.632$, $p = 0.0635$, one-way ANOVA with Tukey's posthoc analysis. 6M: $n_{nTg} = 13$; $n_{TgA53T} = 11$; $n_{TgA53T/mTau^{-/-}} = 10$; $n_{mTau^{-/-}} = 8$. **d.** Top row: heat maps demonstrating time all animals tested spent occupying areas of the YM during the recognition trial. Bottom row: individual trace of representative animal for each group tested during the recognition trial. Orientation of YM is preserved from diagram in **a.** Taken together, this experiment demonstrates that TgA53T mice present with tau-dependent deficits in spatial learning and memory via YM prior to developing deficits that can be detected via Barnes Maze. t test and one-way ANOVA: ** $p < 0.01$ and **** $p < 0.0001$. ns: not significant. Error bars represent mean \pm S.E.M.

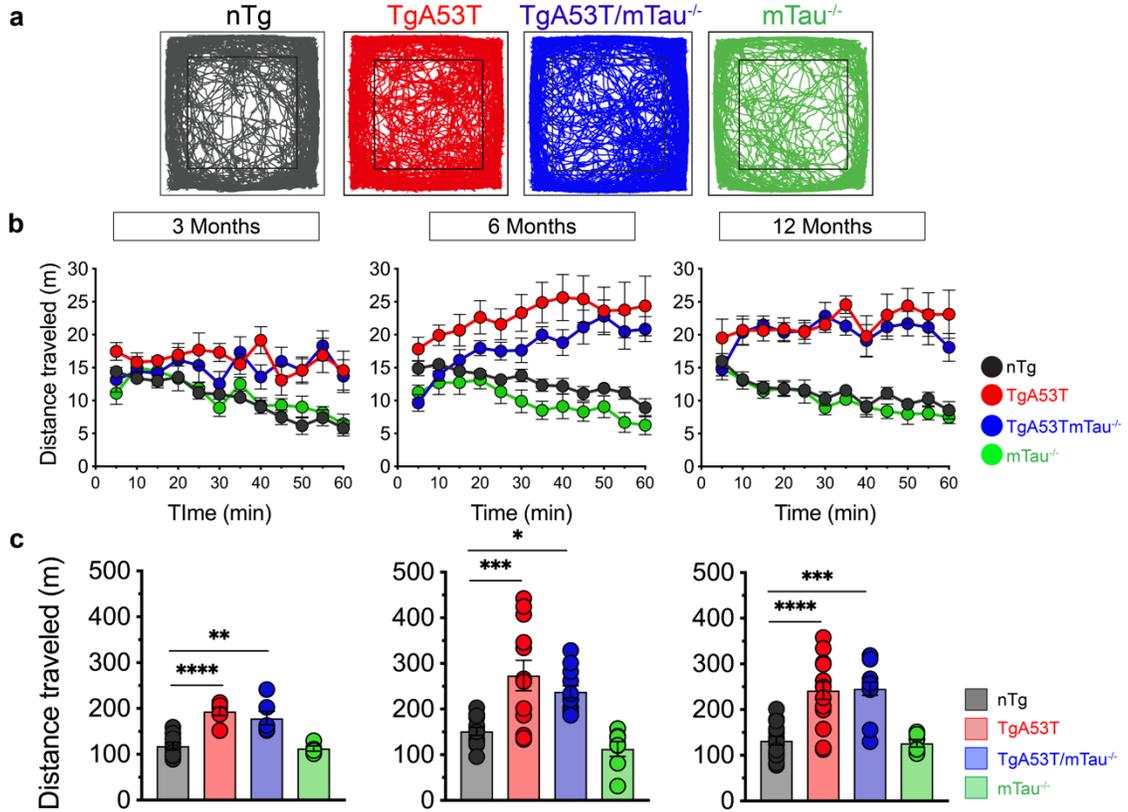


Figure 2.7 – Locomotor hyperactivity in the TgA53T model is tau-independent. **a.** Distance traveled (meters), binned into 5-minute intervals, for the entire 60-minute activity trial. **b.** Summary graph of total distance traveled during the entire 60-minute trial. TgA53T and TgA53T/mTau^{-/-} mice exhibit increases activity compared to controls (nTg and mTau^{-/-}). 3 months (3M): $F_{(3,21)} = 13.81$ ($p < 0.0001$), one-way ANOVA with Tukey's posthoc analysis. 6 months (6M): $F_{(3,40)} = 11.47$ ($p < 0.0001$), one-way ANOVA with Tukey's posthoc analysis. 12 months (12M): $F_{(3,47)} = 19.28$ ($p < 0.0001$), one-way ANOVA with Tukey's posthoc analysis. 3M: $n_{nTg} = 9$; $n_{TgA53T} = 6$; $n_{TgA53T/mTau^{-/-}} = 6$; $n_{mTau^{-/-}} = 5$. 6M: $n_{nTg} = 11$; $n_{TgA53T} = 12$; $n_{TgA53T/mTau^{-/-}} = 11$; $n_{mTau^{-/-}} = 9$. 12M: $n_{nTg} = 13$; $n_{TgA53T} = 9$; $n_{TgA53T/mTau^{-/-}} = 11$; $n_{mTau^{-/-}} = 8$. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Error bars represent mean \pm S.E.M.

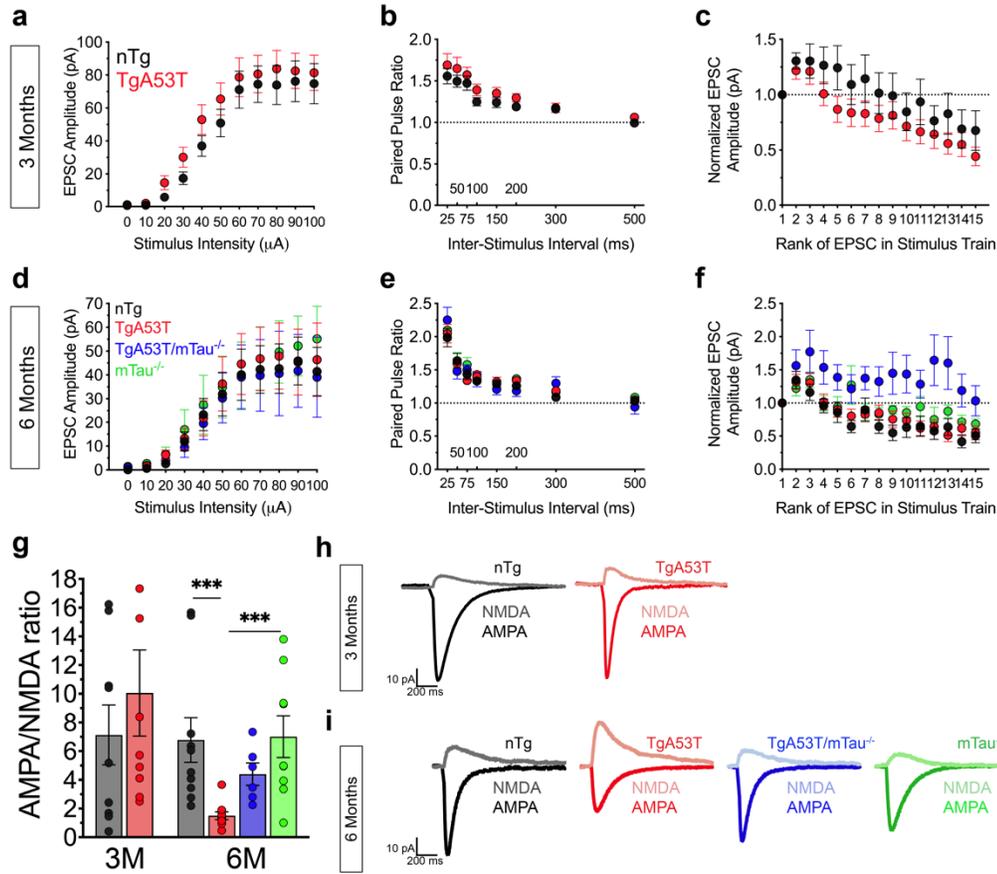


Figure 2.8 – Tau is required for progressive loss of AMPAR-mediated neurotransmission in TgA53T neurons. Core excitatory postsynaptic current (EPSC) synaptic parameters assessed in acute hippocampal slices from animals at 2-3 (3 Months, 3M) and 5-6 (6 Months, 6M) months of age, respectively: **a and d.** Input-output curve. **b and e.** Short-term potentiation measured via paired-pulse facilitation. **c and f.** Short-term depression analyzed through synaptic fatigue. 3M: Input-output: genotype $F_{(1,262)} = 4.790$, $p = 0.0295$, stimulus intensity $F_{(10,262)} = 28.19$, $p < 0.0001$, genotype*stimulus intensity interaction $F_{(10,262)} = 0.1596$, $p = 0.9985$; Paired-pulse: genotype $F_{(1,176)} = 7.422$, $p = 0.0071$; inter-stimulus interval $F_{(7,176)} = 15.34$, $p < 0.0001$; genotype*inter-stimulus interval interaction $F_{(7,176)} = 0.1785$, $p = 0.9894$; Synaptic fatigue: genotype $F_{(1,360)} = 14.26$, $p = 0.0002$, EPSC in train $F_{(14,360)} = 4.611$, $p < 0.0001$, genotype*EPSC in train interaction $F_{(14,360)} = 0.2468$, $p = 0.9978$; all two-way ANOVA with Sidak's posthoc analysis. 6M: Input-output: genotype $F_{(3,407)} = 0.7457$, $p = 0.5253$, stimulus intensity $F_{(10,407)} = 13.90$, $p < 0.0001$, genotype*stimulus intensity interaction $F_{(30,407)} = 0.05744$, $p > 0.9999$; Paired-pulse: genotype $F_{(3,318)} = 0.8501$, $p = 0.4674$, inter-stimulus interval $F_{(7,318)} = 51.83$, $p < 0.0001$, genotype*inter-stimulus interval interaction $F_{(7,318)} = 0.7178$, $p = 0.8147$; Synaptic fatigue: genotype $F_{(3,645)} = 42.33$, $p < 0.0001$, EPSC in train $F_{(14,645)} = 5.048$, $p < 0.0001$, genotype*EPSC in train interaction $F_{(14,645)} = 0.7835$, $p = 0.8360$; all two-way ANOVA with Tukey's posthoc analysis. 3M (mice/slices/cells): $n_{nTg} = 3/7/13$; $n_{TgA53T} = 3/7/13$. 6M (mice/slices/cells): $n_{nTg} = 5/10/11$; $n_{TgA53T} = 5/9/11$; $n_{TgA53T/mTau-/-} = 4/8/8$; $n_{mTau-/-} = 4/11/11$. Except for modest reductions in synaptic fatigue associated with the $mTau^{-/-}$ genotype (**f**), there are no obvious differences in evoked synaptic parameters. **g.** Ratio of amplitude of AMPA:NMDA currents in CA1 pyramidal neurons from nTg and TgA53T mice at 3M, and nTg, TgA53T, TgA53T/ $mTau^{-/-}$, and $mTau^{-/-}$ at 6M. AMPA/NMDA: $F_{(3,31)} = 5.044$, $p = 0.0058$, by one-way ANOVA with Tukey's posthoc analysis. 3M (mice/slices/cells): $n_{nTg} = 3/6/9$ cells; $n_{TgA53T} = 3/6/9$ cells. 6M (mice/slices/cells): $n_{nTg} = 7/9/10$ cells; $n_{TgA53T} = 4/10/10$ cells; $n_{TgA53T/mTau-/-} = 3/7/7$ cells; $n_{mTau-/-} = 3/8/9$ cells. Example AMPA and NMDA current traces from 3M (**h**) and 6M groups (**i**). While the AMPA/NMDA ratio is normal in 3-month-old TgA53T neurons, there is significant reduction in 6-month-old neurons. t test, and one- and two-way ANOVA: * $p < 0.05$ and *** $p < 0.001$. Error bars represent mean \pm S.E.M. Contributions: AC and CN.

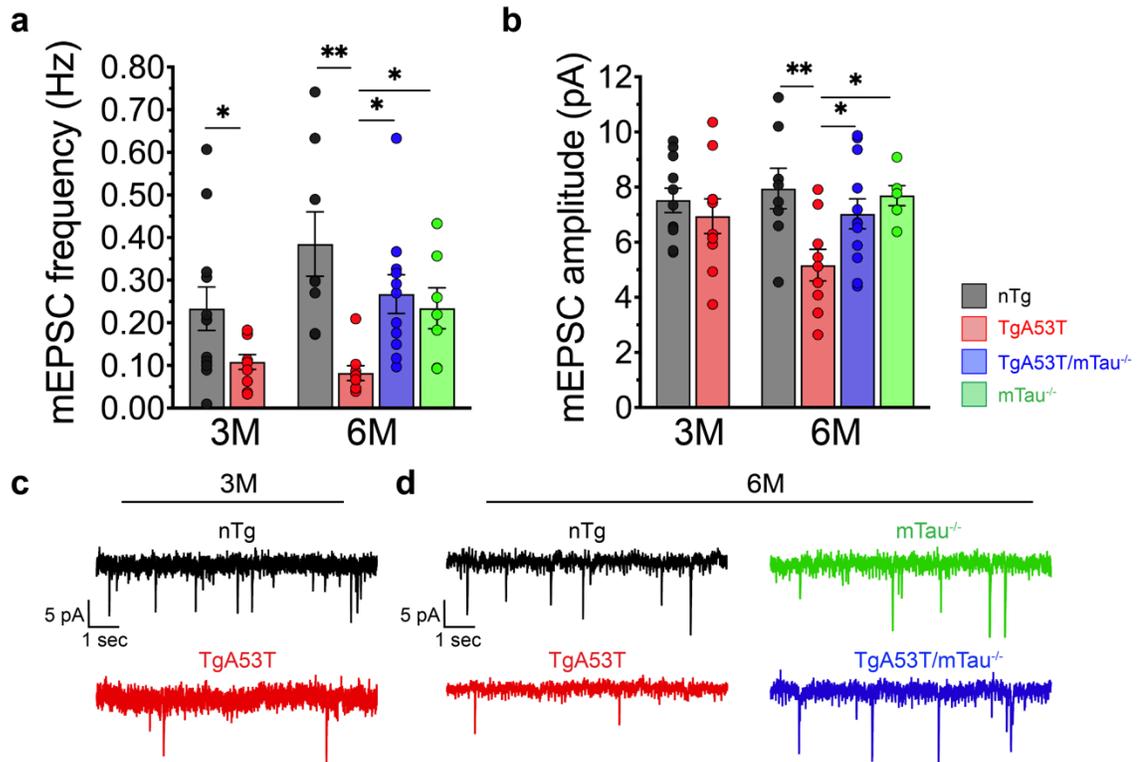


Figure 2.9 – Spontaneous synaptic activity deficits in TgA53T neurons are reversed by loss of mTau expression. Spontaneous recordings of mini excitatory postsynaptic currents (mEPSCs) from CA1 pyramidal neurons in acute hippocampal slices were recorded from and analyzed for frequency (**a**) and amplitude (**b**) of mEPSCs from mice at 2-3 months (3M) and 5-6 months (6M) of age. 3M mEPSC frequency: $t = 2.32$, $df = 13.51$, $p = 0.0364$, by unpaired t test with Welch's correction. 6M mEPSC frequency: $F_{(3, 31)} = 6.213$, $p = 0.0020$, by one-way ANOVA and Tukey's posthoc analysis. 6M mEPSC amplitude: $F_{(3, 31)} = 4.187$, $p = 0.0134$, by one-way ANOVA and Tukey's posthoc analysis. **c.** Example mEPSC traces from 2-3-month-old TgA53T and nTg littermate controls. 3M (mice/slices/cells): $n_{nTg} = 3/7/11$; $n_{TgA53T} = 3/6/10$. 6M (mice/slices/cells): $n_{nTg} = 5/8/8$ cells; $n_{TgA53T} = 3/7/9$ cells; $n_{TgA53T/mTau^{-/-}} = 5/11/12$ cells; $n_{mTau^{-/-}} = 3/6/7$ cells. **d.** Example mEPSC traces from 5-6-month-old nTg, TgA53T, TgA53T/mTau^{-/-}, and mTau^{-/-} mice. The results show that reductions in mEPSC frequency in TgA53T neurons is not progressive from 3M to 6M, while reductions in mEPSC amplitude in TgA53T neurons is age-progressive over this time frame. t test and one-way ANOVA: * $p < 0.05$ and ** $p < 0.01$. Error bars represent mean \pm S.E.M. Contributions: AC and CN.

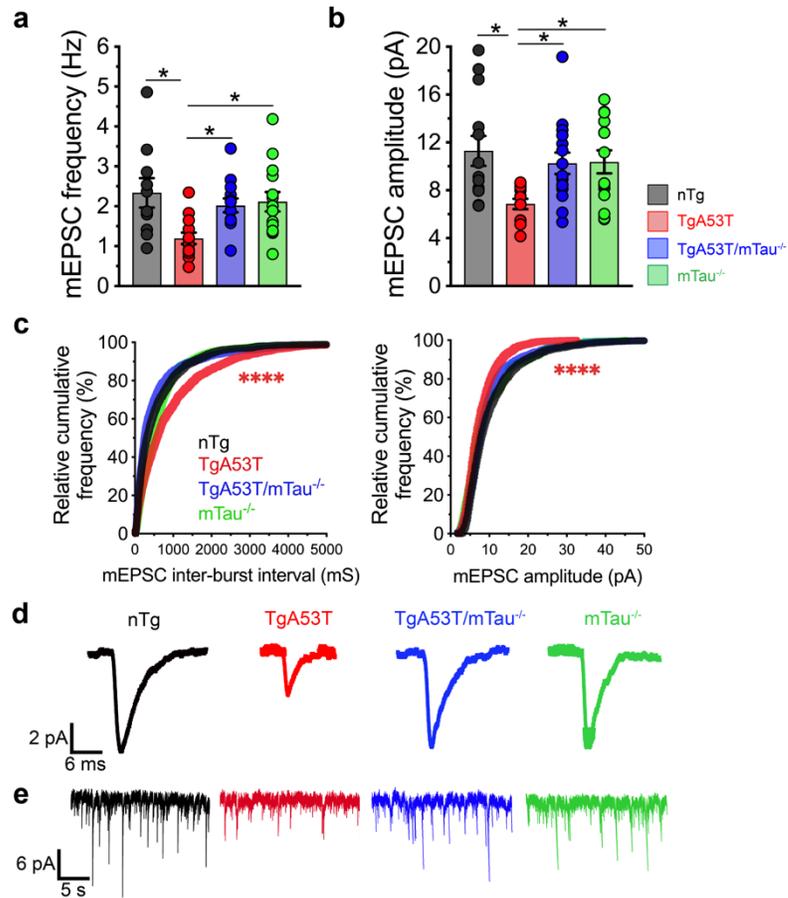


Figure 2.10 – Tau is required for synaptic deficits in cultured TgA53T neurons. Average mini excitatory post-synaptic current (mEPSC) frequency (**a**) and amplitude (**b**) from *in vitro* recordings utilizing mouse primary hippocampal neuronal cultures. Each point represents average mEPSC frequency or amplitude from recording of a single neuron, with neurons coming from multiple litters (cultures). mEPSC frequency: $F_{(3,50)} = 3.872$, $p = 0.0144$, by one-way ANOVA and Tukey's posthoc analysis. mEPSC amplitude: $F_{(3,46)} = 4.551$, $p = 0.0071$, by one-way ANOVA and Tukey's posthoc analysis. **c**. Relative cumulative distribution frequency (CDF) of whole-cell mEPSC inter-burst interval (frequency, left panel) and amplitudes (right panel) from recordings of cultured neurons. mEPSC inter-burst interval CDF: Kolmogorov-Smirnov test, $D = 0.4681$, $p < 0.0001$. mEPSC amplitude CDF: Kolmogorov-Smirnov test, $D = 0.6042$, $p < 0.0001$. $N = 3$ cultures/genotype, $n_{nTg} = 13$ cells; $n_{TgA53T} = 12$ cells; $n_{TgA53T/mTau^{-/-}} = 15$ cells; $n_{mTau^{-/-}} = 14$ cells. **d and e**. Representative traces of whole-cell mEPSCs recorded from dissociated mouse primary hippocampal neuronal cultures. mEPSC recordings from dissociated hippocampal neuron cultures demonstrate that both pre and postsynaptic deficits TgA53T neurons are tau-dependent. One-way ANOVA: * $p < 0.05$, **** $p < 0.0001$. Error bars represent mean \pm S.E.M. Contributions: PJT.

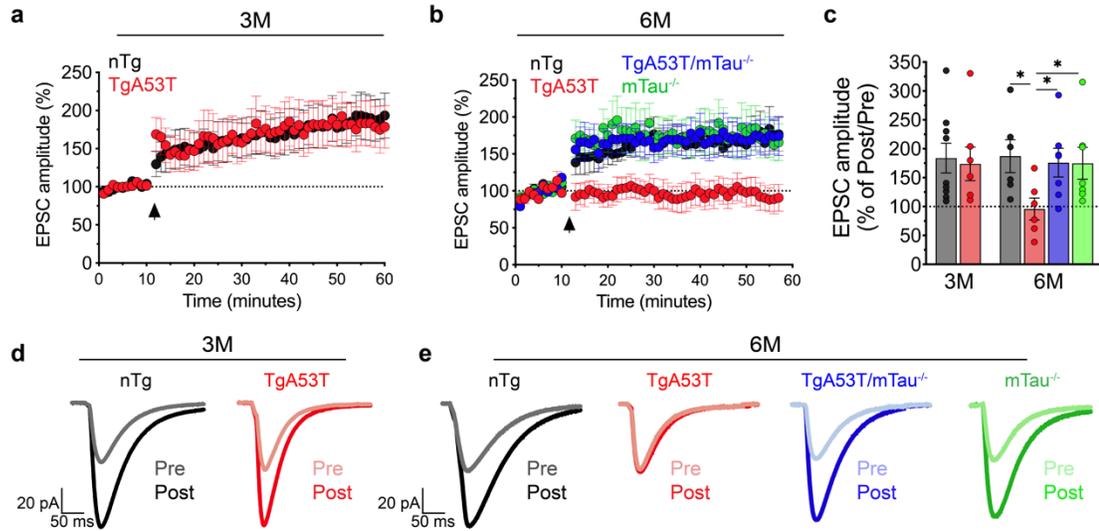


Figure 2.11 – Progressive deficits in long-term potentiation in TgA53T neurons are tau-dependent and correlate with onset of cognitive impairments. a and b. Excitatory postsynaptic currents (EPSC) recorded via whole cell recordings from hippocampal CA1 pyramidal neurons during long-term potentiation induced by high frequency stimulation (HFS) of Schaffer collaterals of animals at 2-3 months (3M) (a) and 5-6 months (6M) of age (b). Arrowhead indicates application of HFS. **c.** EPSC amplitudes 45 minutes following HFS (Post), relative to baseline established prior to HFS (Pre), from both 3M and 6M animals. 6M LTP EPSC: $F_{(3,22)} = 2.584$, $p = 0.0262$, by one-way ANOVA and Tukey's posthoc analysis. 3M (mice/slices/cells): $n_{nTg} = 4/9/9$ cells; $n_{TgA53T} = 4/7/7$ cells. 6M (mice/slices/cells): $n_{nTg} = 5/6/6$ cells; $n_{TgA53T} = 5/6/6$ cells; $n_{TgA53T/mTau^{-/-}} = 3/7/7$ cells; $n_{mTau^{-/-}} = 3/7/7$ cells. These results show that TgA53T neurons exhibit normal LTP at 3M but severe LTP deficits at 6M. Further, TgA53T/mTau^{-/-} neurons exhibit normal LTP at 6M. **d and e.** Example EPSC traces from LTP experiments. Presented here are Pre and Post HFS in 3M neurons (d) and 6M neurons (e). t test and one-way ANOVA: * $p < 0.05$. Error bars represent mean \pm S.E.M. Contributions: AC and CN.

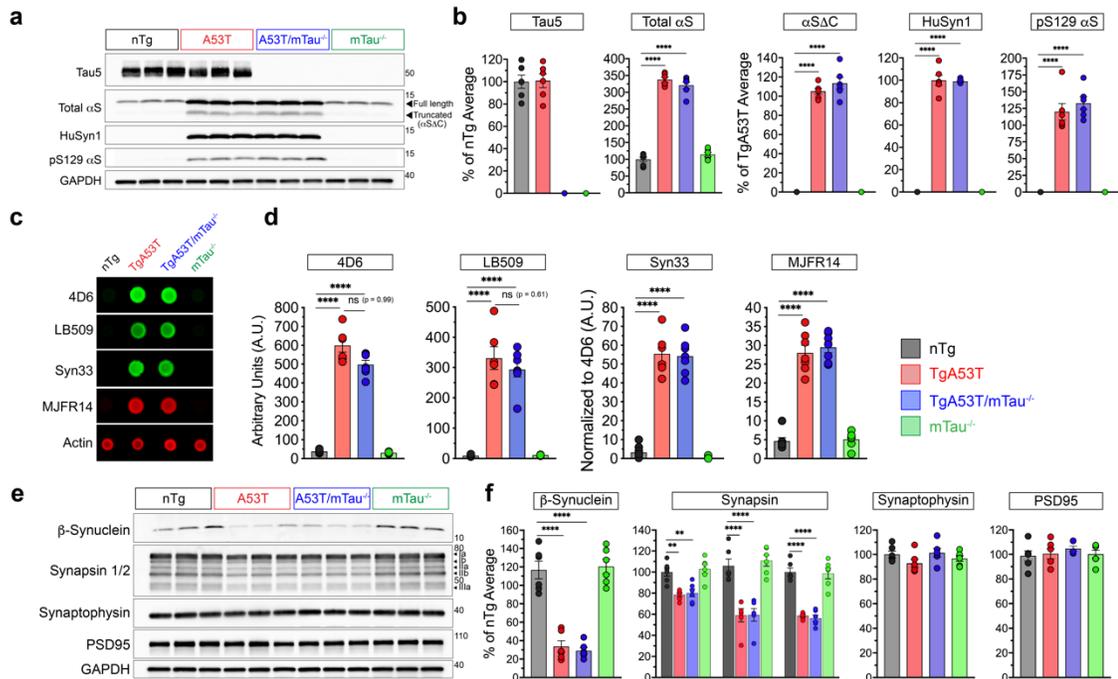


Figure 2.12 – Tau-dependent synaptic and cognitive deficits in TgA53T mice are independent of expression or aggregate-specific changes in α S or key presynaptic proteins. **a.** Representative western blot analysis of hippocampal lysates from 12-month-old mice. **b.** Densitometry of hippocampal protein expression. For tau and total α S (full length), values were normalized to the average values for nTg samples within each gel. For truncated α S (α S \square C), human α S (HuSyn1), α S phosphorylated at Ser129 (pS129 α S), values were normalized to the average densitometric values of TgA53T samples within each gel. For western blot densitometry: one-way ANOVA with Tukey's posthoc analysis. Total α S: $F_{(3,20)} = 252.4$, $p < 0.0001$. α S \square C: $F_{(3,20)} = 335.9$, $p < 0.0001$. HuSyn1: $F_{(3,20)} = 616.2$, $p < 0.0001$. pS129 α S: $F_{(3,20)} = 88.70$, $p < 0.0001$. While α S-associated protein levels are increased in TgA53T mice, the levels are not altered in TgA53T/mTau^{-/-} mice. N = 6 animals/genotype. **c.** Representative dot blots from non-denatured 12-month-old hippocampal lysates for the epitopes associated with total α S (4D6), human α S (LB509), and various pathological α S oligomers (Syn33, MJFR14). Dot blots for additional α S oligomers and pathological tau are shown in Suppl. Figure 5 (Online Resources). **d.** Dot blot densitometry for levels of total α S (4D6) and human α S (LB509), normalized to actin levels. For higher order α S species, Syn33 and MJFR14, densitometry values were normalized to the average densitometric values of TgA53T samples within each gel. For all dot blot densitometry: one-way ANOVA with Tukey's posthoc analysis. 4D6: $F_{(3,23)} = 232.9$, $p < 0.0001$. LB509: $F_{(3,23)} = 68.53$, $p < 0.0001$. Syn33: $F_{(3,23)} = 116.4$, $p < 0.0001$. MJFR14: $F_{(3,23)} = 101.1$, $p < 0.0001$. N = 8 animals/genotype. While α S and α S oligomer species levels are increased in TgA53T mice, they are unchanged by tau removal in TgA53T/mTau^{-/-} mice. **e.** Representative western blot images of presynaptic and postsynaptic proteins of interest in 12-month-old hippocampi. **f.** Densitometry of hippocampal protein expression. Values were normalized to the average values for nTg samples within each gel. For western blot densitometry: one-way ANOVA with Tukey's posthoc analysis. β -synuclein: $F_{(3,20)} = 49.86$, $p < 0.0001$. Synapsin Ia+b: $F_{(3,20)} = 8.501$, $p = 0.0008$. Synapsin IIa+b: $F_{(3,20)} = 9.482$, $p = 0.0004$. Synapsin IIIa: $F_{(3,20)} = 46.62$, $p < 0.0001$. N = 6 animals/genotype. Densitometry shows that while β -synuclein and synapsin isoforms are decreased in TgA53T mice, they are not altered by loss of tau expression. Further, the levels of synaptophysin and PSD95 are comparable in all animals, indicating a lack of synaptic loss. For all, values were normalized to the average densitometric values of nTg samples within each gel. One-way ANOVA: ** $p < 0.01$ and **** $p < 0.0001$. ns: not significant. Error bars represent mean \pm S.E.M. Contributions: MAB and SEL.

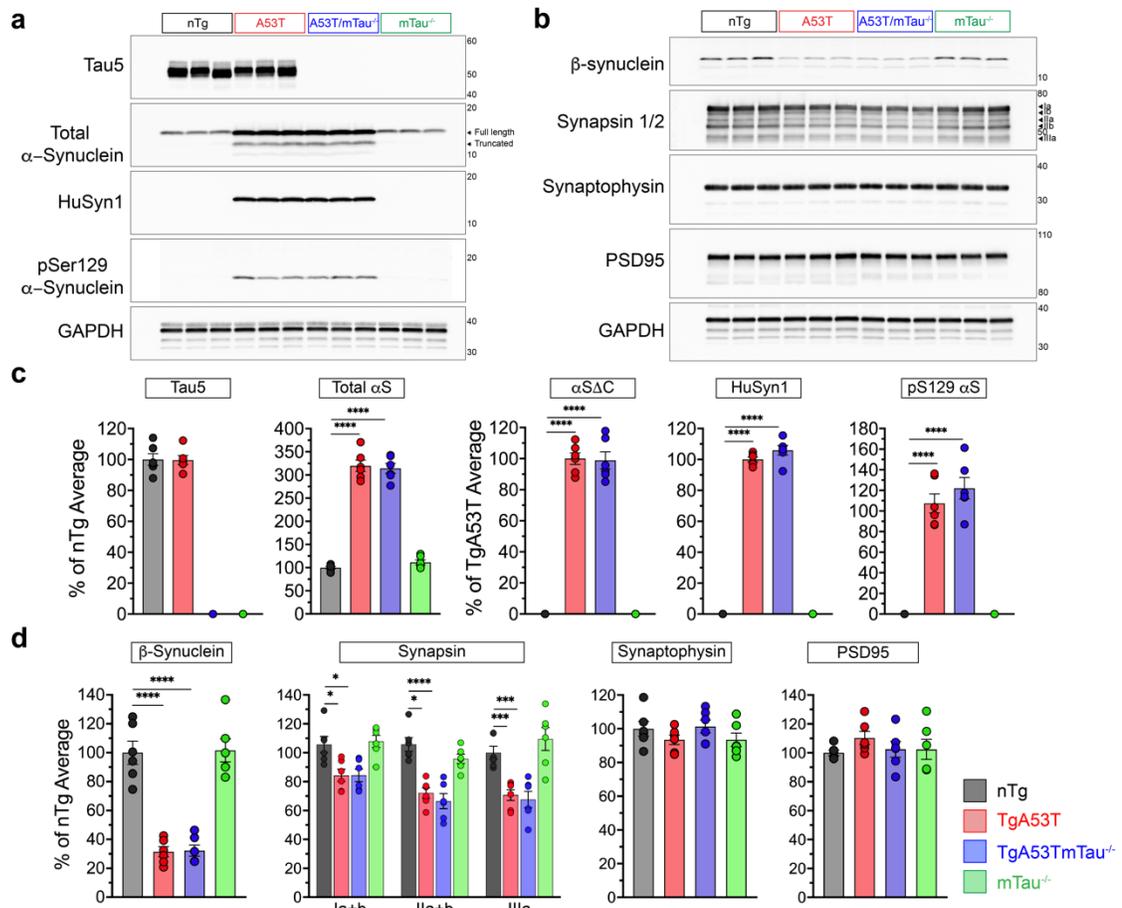


Figure 2.13 – Cortical levels of α S expression, α S post-translational modifications, and synaptic proteins are tau-independent. **a** and **b**. Representative western blot analysis of cortical lysates from 12-month-old mice probing for total tau or α S (**a**), or key pre and postsynaptic proteins of interest (**b**). **c**. Densitometry of cortical protein expression. For tau and total α S, values were normalized to the average values for nTg samples within each gel. For truncated α S (α S Δ C), HuSyn1 (human α S), α S phosphorylated at Ser129 (pSer129- α S, pS129 α S), values were normalized to the average densitometric values of TgA53T samples within each gel. For all densitometry: one-way ANOVA with Tukey's posthoc analysis. Total α S: $F_{(3,20)} = 198.9$, $p < 0.0001$. α S Δ C: $F_{(3,20)} = 298.2$, $p < 0.0001$. HuSyn1: $F_{(3,20)} = 1163.0$, $p < 0.0001$. pS129 α S: $F_{(3,20)} = 91.72$, $p < 0.0001$. **d**. Synapse densitometry, with values normalized GAPDH and compared to average of nTg samples. For western blot densitometry: one-way ANOVA with Tukey's posthoc analysis. β -synuclein: $F_{(3,20)} = 41.07$, $p < 0.0001$. Synapsin Ia+b: $F_{(3,20)} = 9.161$, $p = 0.0005$. Synapsin IIa+b: $F_{(3,20)} = 9.794$, $p = 0.0003$. Synapsin IIIa: $F_{(3,20)} = 13.70$, $p < 0.0001$. $N = 6$ animals/genotype. Densitometry shows that while β -synuclein and synapsin isoforms are decreased in TgA53T mice, they are not altered by loss of tau expression. Further, the levels of synaptophysin and PSD95 are comparable in all animals, indicating a lack of synaptic loss. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$. Error bars represent mean \pm S.E.M.

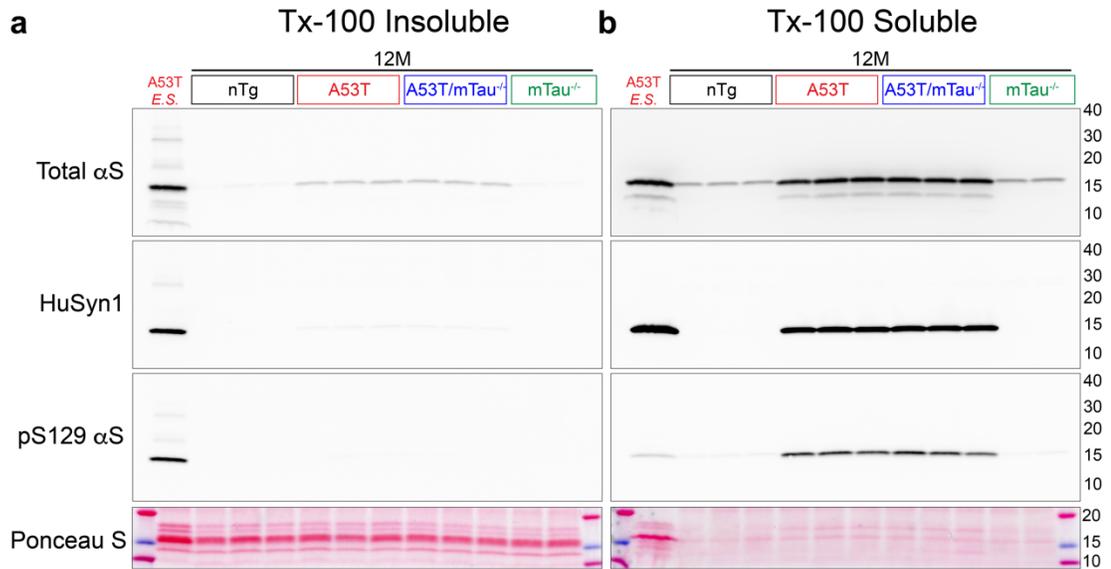


Figure 2.14 – Detergent-insoluble α S aggregates accumulate in symptomatic TgA53T animals in a tau-independent fashion. a and b. Nonionic detergent- (TritonTM X-100, TX) fractionation of insoluble and soluble components from 12M mice. Representative western blot images for levels and species of total α S, human α S (Syn101), and α S phosphorylated at serine 129 (pS129 α S) from the detergent-insoluble (**a**) and soluble fractions (**b**). Ponceau S staining of nitrocellulose membranes post-transfer demonstrates equal protein loading and consistent transfer across all lysates. The accumulation of nonionic detergent-insoluble α S species occurs exclusively in pathologically-affected brain regions of symptomatic “end-stage” (E.S.) TgA53T animals, particularly the brain stem and spinal cord. As a positive control, the A53T E.S. soluble and insoluble lysate fractions were prepared from the brain stem of an end stage TgA53T animal. Given the modest and comparable levels of insoluble α S in asymptomatic 12M TgA53T and TgA53T/mTau^{-/-} animals, as well as the comparable levels of soluble α S levels in these groups, it suggests that soluble pathogenic species of α S are likely mediators of the h α S^{A53T}-driven synaptic and memory deficits. (n_{TgA53T-E.S.} = 1, n_{nTg} = 3, n_{TgA53T} = 3, n_{TgA53T/mTau^{-/-}} = 3, n_{mTau^{-/-}} = 2).

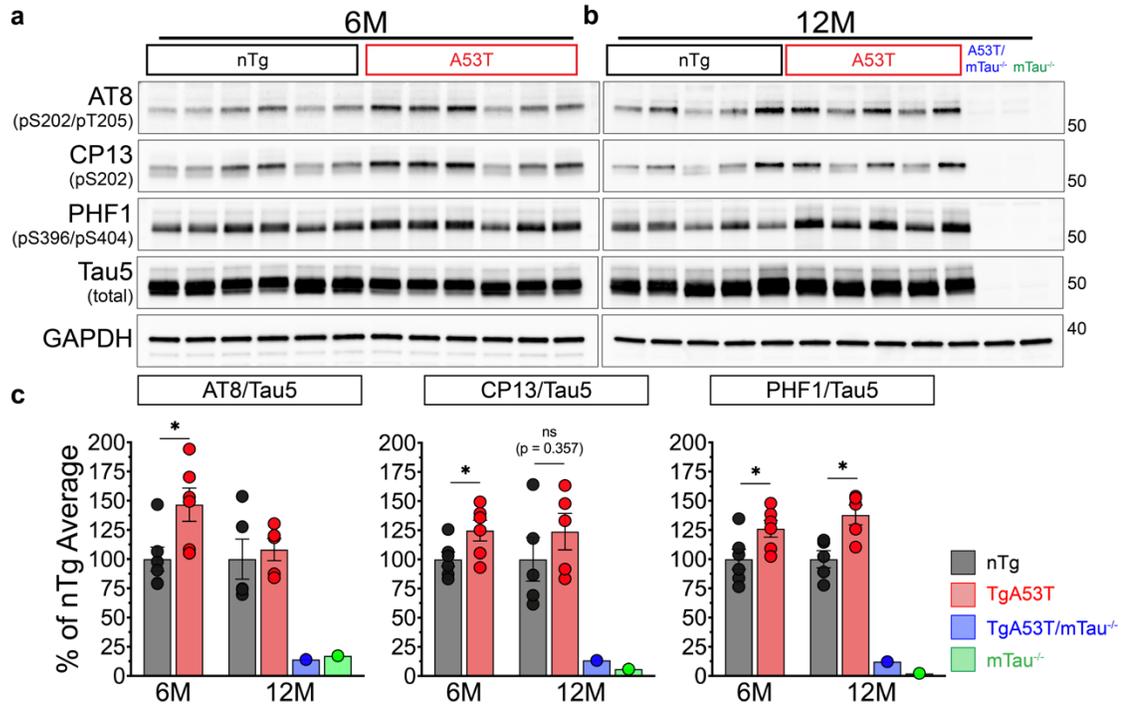


Figure 2.15 –Synaptic and cognitive dysfunction in TgA53T are associated with modest but significant increases in pathological phosphorylated species of tau. a and b. Representative western blot analysis of hippocampal lysates from 6-month-old (6M) (a) 12-month-old (12M) (b) mice probing for disease-associated phosphorylated species of tau (AT8, CP13, and PHF1). **c.** Densitometry quantifying phosphorylated tau protein expression in the hippocampus. For 6M and 12M densitometry, expression of these pathological species of tau was normalized to total tau (Tau5) levels within each hippocampi, and then analyzed by unpaired t test with Welch’s correction between nTg and TgA53T lysate values. AT8_{6M}: $t = 2.656$, $df = 9.115$, $p = 0.260$. CP13_{6M}: $t = 2.286$, $df = 9.050$, $p = 0.0479$. PHF1_{6M}: $t = 2.340$, $df = 9.659$, $p = 0.0422$. 6M: $n_{nTg} = 6$; $n_{TgAA53T} = 6$. AT8_{12M}: $t = 0.4183$, $df = 6.199$, $p = 0.06899$. CP13_{12M}: $t = 0.9803$, $df = 7.750$, $p = 0.3566$. PHF1_{12M}: $t = 23.353$, $df = 7.844$, $p = 0.0103$. 12M: $n_{nTg} = 5$; $n_{TgAA53T} = 5$; $n_{TgA53T/mTau^{-/-}} = 1$; $n_{mTau^{-/-}} = 1$. t test: * $p < 0.05$. ns: not significant. Error bars represent mean \pm standard error of the mean (S.E.M).

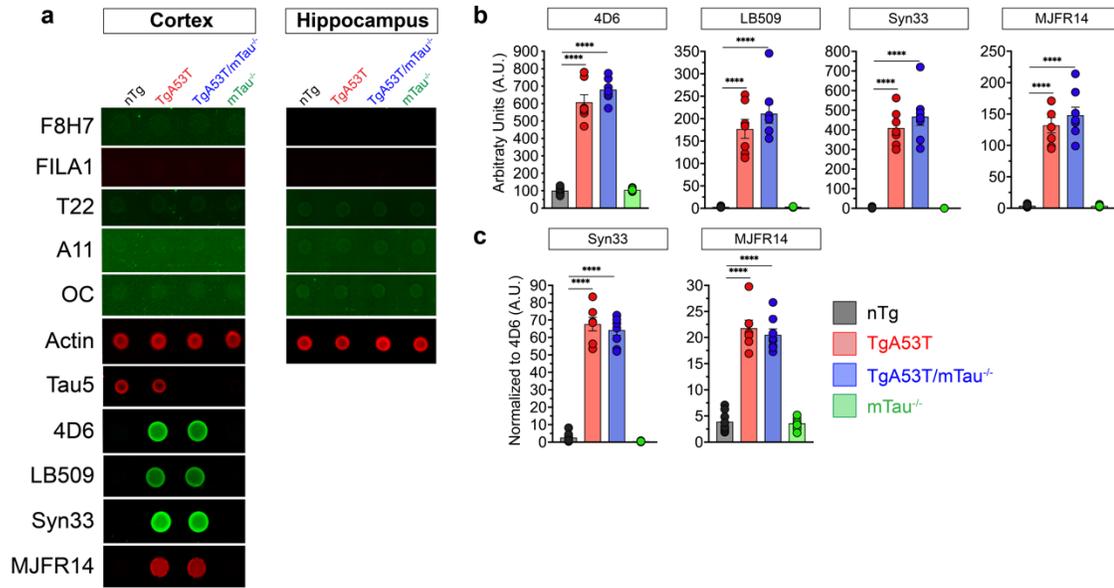


Figure 2.16 – Synaptic and cognitive deficits in TgA53T mice are independent of expression or oligomeric-specific changes in α S or tau. **a.** Representative dot blots of species of α S and tau in non-denatured cortical and hippocampal lysates from 12-month-old mice (12M). The antibodies used here recognize epitopes associated with total α S (4D6), human α S (LB509), various pathological α S oligomers (F8H7, FILA1, Syn33, MJFR14), total tau (Tau5), pathological tau oligomers (T22), pan soluble oligomers (A11), and amyloid fibrils (OC). Antibody details can be found in Suppl. Table 1 (Online Resources). **b.** Densitometry of cortical protein expression normalized to the average densitometric values of nTg samples within each age. Only epitopes showing significant signal or increases in TgA53T mice were analyzed. For dot blot densitometry: one-way ANOVA with Tukey's posthoc analysis. 4D6: $F_{(3,27)} = 191.0$, $p < 0.0001$. LB509: $F_{(3,27)} = 59.12$, $p < 0.0001$. Syn33: $F_{(3,27)} = 86.28$, $p < 0.0001$. MJFR14: $F_{(3,27)} = 84.98$, $p < 0.0001$. **c.** Densitometry quantifying higher order synuclein species expression (Syn33 and MJFR14), normalized to the average total α S (4D6) within genotype. For dot blot densitometry: one-way ANOVA with Tukey's posthoc analysis: Syn33/4D6: $F_{(3,27)} = 246.4$, $p < 0.0001$. MJFR14/4D6: $F_{(3,27)} = 101.1$, $p < 0.0001$. $N = 8$ animals/genotype. Western blotting and densitometry demonstrates that synaptic and memory abnormalities in TgA53T mice are not due to changes in insolubility of α S or protein expression of α S but may occur downstream or independent of pathological α S species, in a tau-dependent manner. One-way ANOVA: **** $p < 0.0001$. Error bars represent mean \pm S.E.M. Contributions: MAS and SEL.

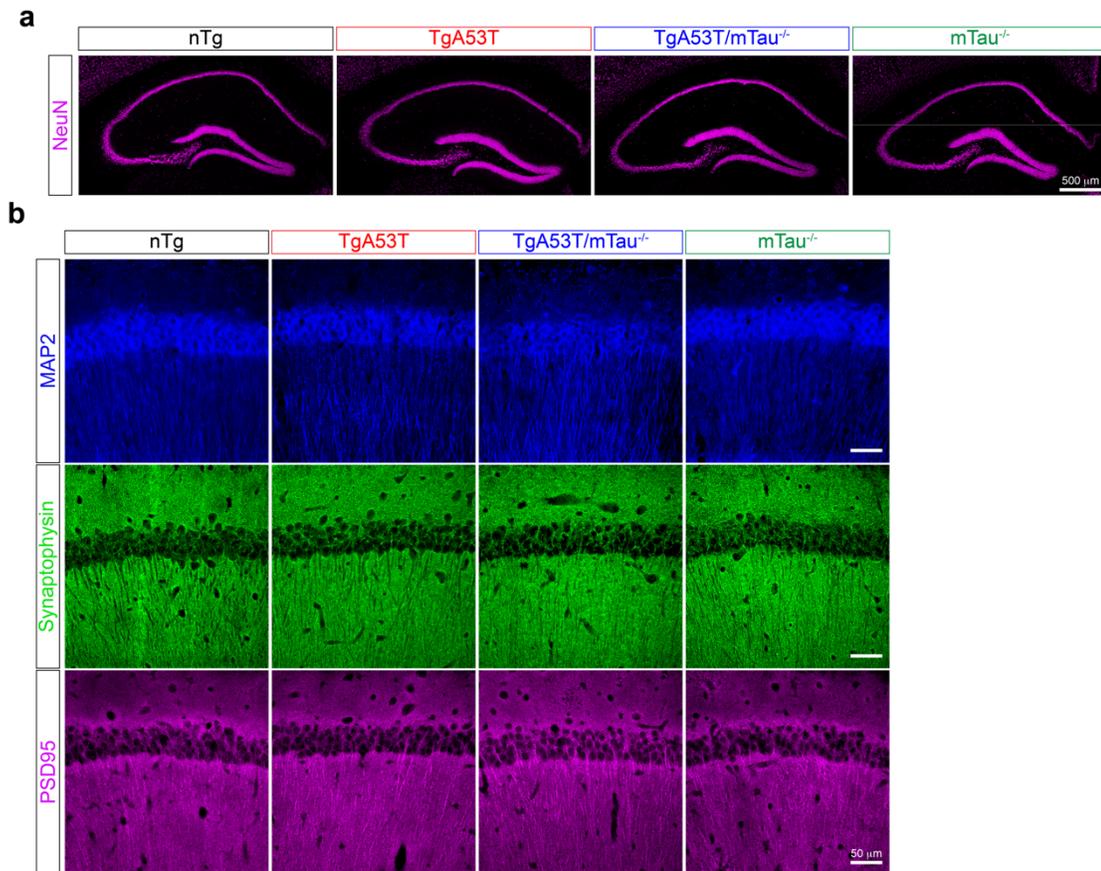


Figure 2.17 – Neither synapse nor neuronal loss are required for synaptic dysfunction and memory deficits in TgA53T mice. a and b. Confocal images of immunohistochemical staining on 12-month-old hippocampi from nTg, TgA53T, TgA53T/mTau^{-/-}, and mTau^{-/-} mice. Hippocampi stained for the neuronal nuclei marker NeuN (**a**). Scale bar: 500 μ m. CA1 region of the hippocampus stained for somatodendritic (MAP2), presynaptic (synaptophysin), and postsynaptic (PSD95) structures (**b**). Scale bar: 50 μ m. Qualitatively, the lack of changes in gross hippocampal structure as well as synapse integrity when comparing nTg and TgA53T mice at 12 months of age indicates that synaptic dysfunction, rather than loss, is responsible for h α S^{A53T}-mediated cognitive decline. Contributions: EO.

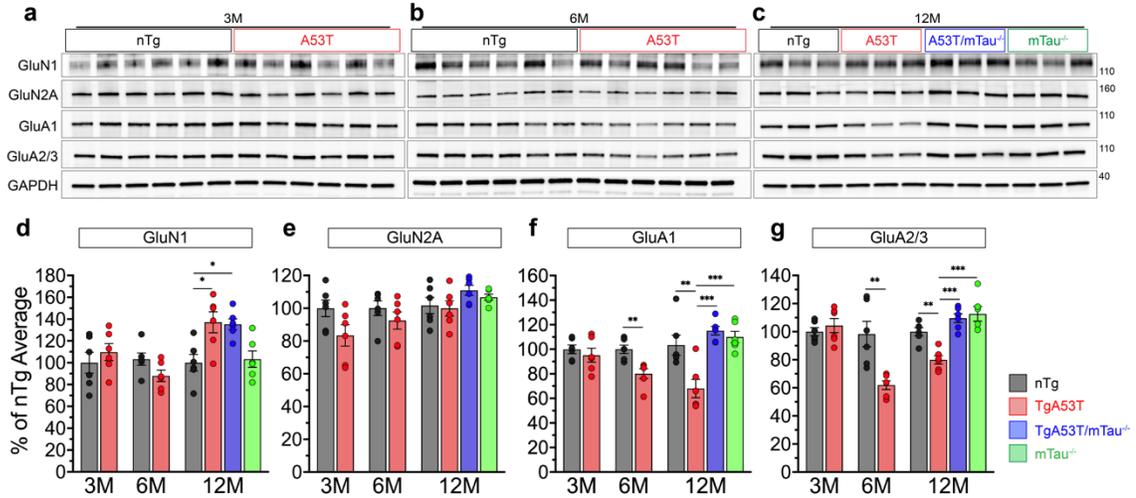


Figure 2.18 – Progressive loss of AMPA receptor subunits in the TgA53T is tau-dependent. Representative western blot images of hippocampal lysates of AMPA (GluA) and NMDA (GluN) receptor subunits at 3 (**a**), 6 (**b**), and 12 (**c**) months of age (3M, 6M, and 12M, respectively). **d-g**. Densitometry of immunoblots for AMPA and NMDA receptor subunits at 3M, 6M, and 12M. For all, values were normalized to the average densitometric values of nTg samples within each gel. For 3M and 6M: unpaired t test with Welch's correction. For 12M densitometry: one-way ANOVA with Tukey's posthoc analysis. 12M GluN1: $F_{(3,20)} = 7.004$, $p = 0.0021$. 6M GluA1: $t = 3.773$, $df = 9.679$, $p = 0.0039$. 12M GluA1: $F_{(3,20)} = 11.55$, $p = 0.0001$. 6M GluA2/3: $t = 3.801$, $df = 6.180$, $p = 0.0085$. 12M GluA2/3: $F_{(3,20)} = 16.21$, $p < 0.0001$. $N = 6$ animals/age/genotype. Compared to nTg mice, the NMDA receptor subunits are not decreased in TgA53T mice at all ages tested (**d and e**). However, AMPA receptor subunits are significantly decreased starting at 6M in TgA53T mice compared to nTg (**f and g**). Significantly, the loss of AMPA receptor subunits are reversed in TgA53T/mTau^{-/-} animals (**f and g**). t test and one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Error bars represent mean \pm S.E.M.

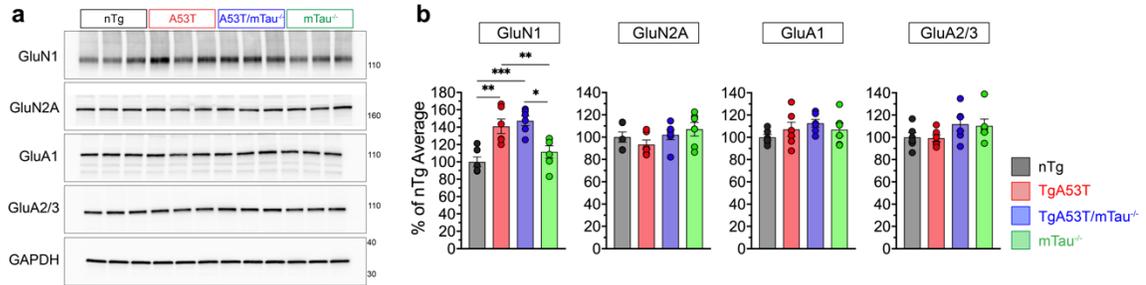


Figure 2.19 – Tau-dependent biochemical changes in postsynaptic glutamatergic (AMPA and NMDA) receptors are region-specific. **a.** Representative western blot images for AMPAR (GluA) and NMDAR (GluN) subunits of cortical lysates from 12-month-old animals. **b.** Densitometry quantifying protein expression in isolated cortices. For all, values were normalized to the average densitometric values of nTg samples within each gel. For all densitometry: one-way ANOVA with Tukey’s posthoc analysis. GluN1: $F_{(3,20)} = 11.49$, $p < 0.0001$. $N = 6$ animals/genotype. Compared to nTg mice cortices, cortical AMPA and NMDA receptor expression is not decreased in TgA53T mice at 12 months of age, when TgA53T mice have established synaptic deficits, evidence of network abnormalities, and memory loss. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Error bars represent mean \pm S.E.M.

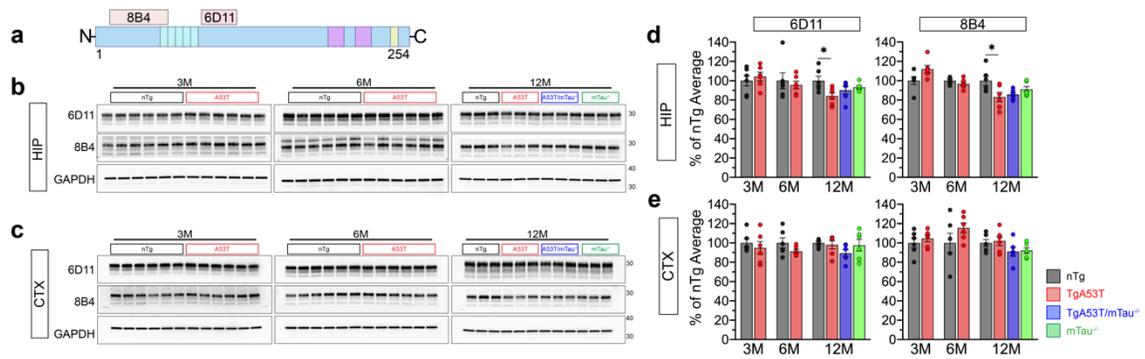


Figure 2.20 – Tau-dependent synaptic dysfunction in TgA53T neurons is not associated with increases in PrP^C expression and signaling. **a.** Graphical depiction of cellular prion protein (PrP^C) primary structure and antibody binding regions. **b and c.** Representative western blot images of hippocampal (**b**) and cortical (**c**) lysates for PrP^C expression at 3, 6 and 12 months of age (3M, 6M, and 12M, respectively). **d and e.** Densitometry of protein expression in hippocampal (HIP) (**d**) and cortical (CTX) (**e**) lysates, normalized to the average densitometric values of nTg samples within each gel. For 3M and 6M densitometry, unpaired t test with Welch's correction. For 12M densitometry: one-way ANOVA with Tukey's posthoc analysis. 12M HIP 6D11: $F_{(3,20)} = 3.193$, $p = 0.0458$. 12M HIP 8B4: $F_{(3,20)} = 3.685$, $p = 0.0292$. $N = 6$ animals/age/genotype. These results demonstrate that activation of PrP^C signaling is not required for the age-dependent synaptic and memory deficits in TgA5T mice. t test and one-way ANOVA: * $p < 0.05$. Error bars represent mean \pm S.E.M.

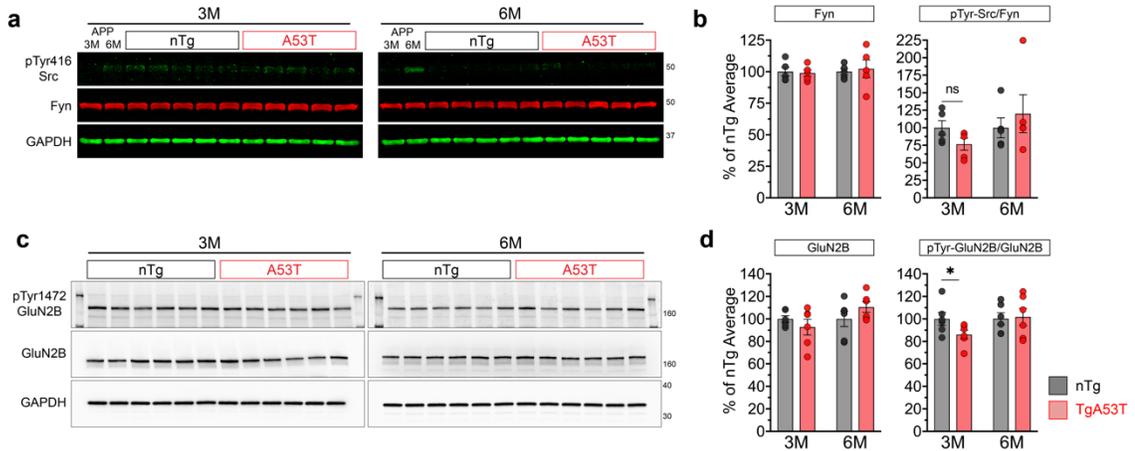


Figure 2.21 – Tau-mediated, mutant α S-induced synaptic and cognitive deficits are independent of postsynaptic Fyn and GluN2B activation. **a and b.** Western blot images for activation status of both Fyn (via pTyr416 Src) (**a**) and NMDA receptor subunit GluN2B (via pTyr1472 GluN2B) (**c**) 3 (left) and 6 (right) months of age (3M and 6M, respectively) through analysis of total and active phosphorylated states. **b and d.** Densitometry quantifying 3M and 6M cortical lysates, normalized to the average densitometric values of nTg samples within each gel. Activation status was determined through examining ratio of values of activating phosphorylation site: pTyr416 for Fyn (**b**) and pTyr1472 for GluN2B (**d**), normalized to respective total levels of protein. For 3M and 6M densitometry, unpaired t test with Welch's correction. 3M pTyr-Src/Fyn: $t = 1.761$, $df = 8$, $p = 0.1163$. $N = 5$ animals/age/genotype. 3M pTyr-GluN2B/GluN2B: $t = 2.539$, $df = 8.066$, $p = 0.0345$. $N = 6$ animals/age/genotype. These findings build on Suppl. Figure 9, further showing that TgA53T mice do not display increased activation of the PrP^C-Fyn-GluN2B signaling cascade as compared to age-matched nTg littermates. Together, these results suggest that $h\alpha$ S^{A53T}-driven and impairments requiring tau are mediated independent of this pathway. t test: * $p < 0.05$. ns: not significant. Error bars represent mean \pm S.E.M. Contributions: MAS and SEL.

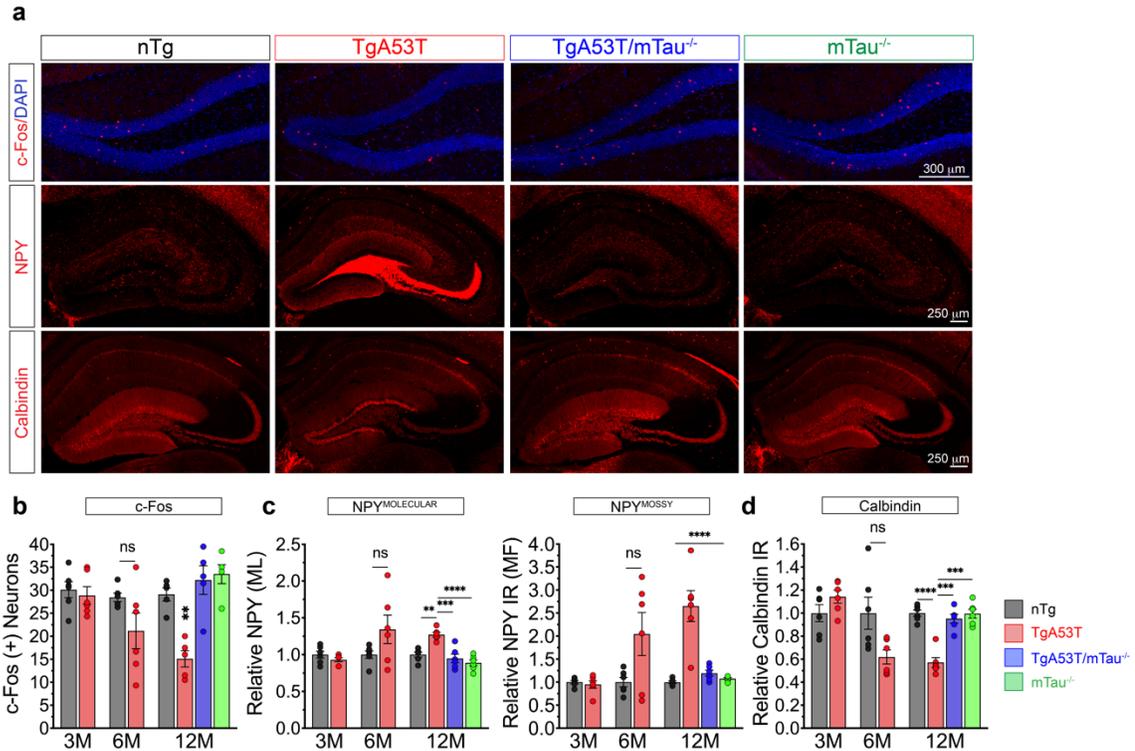


Figure 2.22 – Progressive alterations in inhibitory circuits in the dentate gyrus of TgA53T mice is tau dependent and correlate with the onset of synaptic and memory deficits. a. Representative images from dentate gyri and hippocampi of 12-month-old (12M) nTg, TgA53T, TgA53T/mTau^{-/-}, and mTau^{-/-} mice. Representative images and quantification for 3-month-old (3M) and 6-month-old (6M) are shown in Figure 2.23. c-Fos scale bar: 300 μ m. NPY and calbindin scale bar: 250 μ m. **b-d.** Quantification of immunoreactivity (IR) via cell counting (**b**: c-Fos) or densitometry (**c**: NPY in the Molecular Layer “Molecular”; NPY in the Mossy Fiber pathway, “Mossy”; and, **d**: calbindin) at 3M, 6M, and 12M. 6M: c-Fos: U = 7, p = 0.0844; NPY-Molecular: U = 10, p = 0.2403; NPY-Mossy: U = 11, p = 0.3905; Calbindin: U = 6, p = 0.0649. 12M: c-Fos: $F_{(3,16)} = 14.88$, p < 0.0001; NPY-Molecular: $F_{(3,16)} = 15.97$, p < 0.0001; NPY-Mossy: $F_{(3,16)} = 19.77$, p < 0.0001; Calbindin: $F_{(3,16)} = 19.03$, p < 0.0001. 3M analysis: unpaired t test with Welch’s correction. 6M analysis: Mann-Whitney t test. 12M analysis: one-way ANOVA with Tukey’s posthoc analysis. N = 6 animals/age/genotype, n = 8 sections/animal. In 3M TgA53T mice, the levels of synaptic activity markers are comparable to nTg mice. In 6M TgA53T mice, two distinct populations of mice exist: TgA53T and TgA53T^{INT} (analyzed in Suppl. Figure 11, Online Resources). All 12M TgA53T mice exhibit aberrant network remodeling which was reversed in TgA53T/mTau^{-/-} mice. t test and one-way ANOVA: * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001. ns: not significant. IR: immunoreactivity. ML: molecular layer. MF: mossy fiber. Error bars represent mean \pm S.E.M. Contributions: EO and JM.

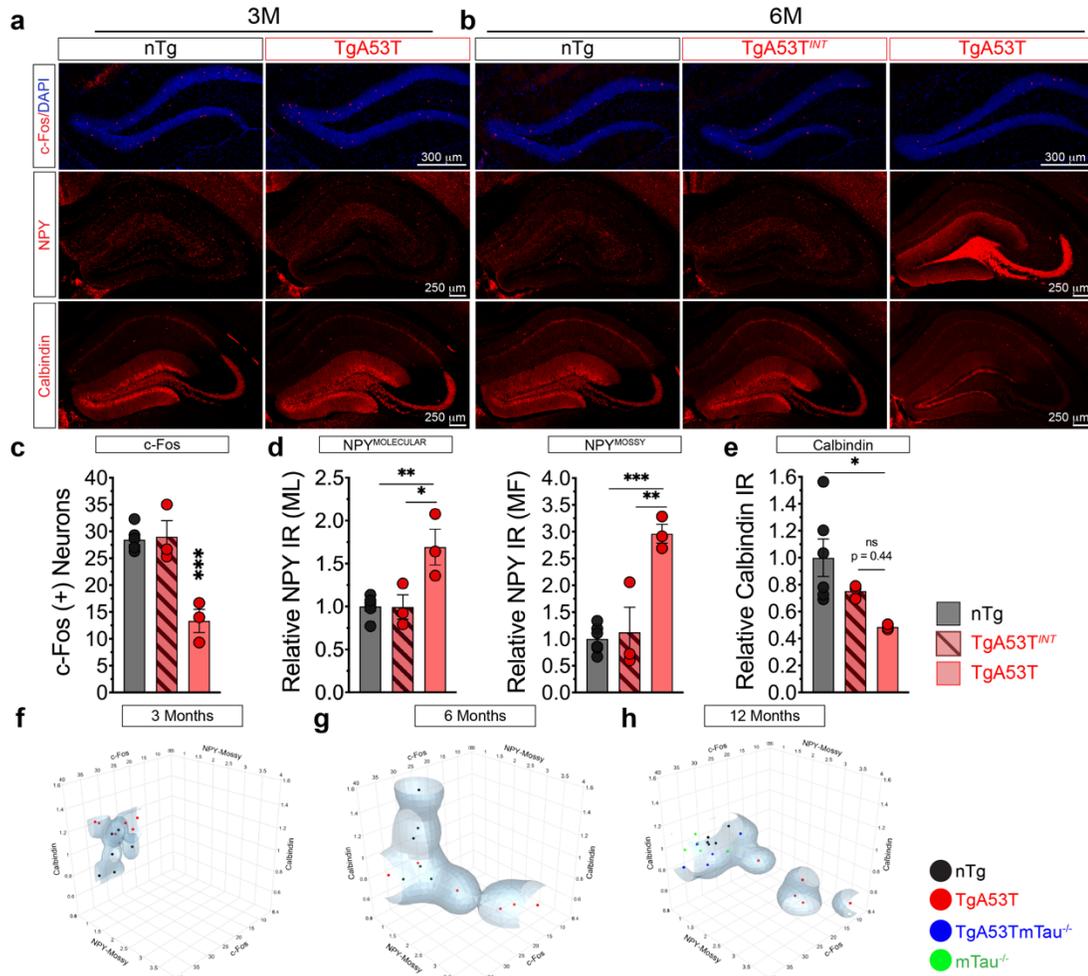


Figure 2.23 – Activity-dependent remodeling of hippocampal circuits is not observed in younger TgA53T mice with intact cognition and appears to follow glutamatergic signaling deficits. **a and b.** Representative confocal images from dentate gyri and hippocampi of 3-month-old (3M, **a**) and 6-month-old (6M, **b**) nTg and TgA53T mice stained for c-Fos, NPY, and calbindin. 3M and 6M c-Fos, NPY, and calbindin quantification values are reported in Fig 9c-e. c-Fos scale bar: 300 μm. NPY and calbindin scale bar: 250 μm. **c-e.** Quantification of immunoreactivity (IR) via cell counting (**c**: c-Fos) or densitometry (**d**: NPY in the Molecular Layer “Molecular”; NPY in the Mossy Fiber pathway, “Mossy”; and, **e**: calbindin) at 6M. 6M TgA53T animals were classified into two groups as some TgA53T mice displayed activity-related changes in c-Fos, calbindin, and NPY while others, the TgA53T^{INT} (for “Intermediate”) resembled their age-matched nTg littermate controls. c-Fos: $F_{(2,9)} = 22.16$, $p = 0.0003$; NPY-Molecular: $F_{(2,9)} = 10.60$, $p = 0.0043$; NPY-Mossy: $F_{(2,9)} = 20.74$, $p = 0.0004$; Calbindin: $F_{(2,9)} = 4.174$, $p = 0.0522$. All by one-way ANOVA with Tukey’s posthoc analysis. $n_{nTg} = 6$; $n_{TgA53T} = 3$; $n_{TgA53T-INT} = 3$. **f-h.** Three-dimensional (3D) X-Y-Z scatterplot of c-Fos, NPY (Mossy Fiber, “NPY-Mossy”), and calbindin immunostaining at 3 (**f**), 6 (**g**), and 12 (**h**) months (3M, 6M, and 12M, respectively). 3M TgA53T mice do not display evidence of network remodeling. The two distinct TgA53T populations present at 6M are clearly observed: one TgA53T group displayed activity-related changes in c-Fos, calbindin, and NPY while others, the TgA53T^{INT} (for “Intermediate”) more closely resembled their age-matched nTg littermate controls. By 12M, all aged TgA53T mice demonstrate signs of chronic hippocampal network hyperactivity. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. ns: not significant. Error bars represent mean \pm S.E.M. Contributions: EO and JM.

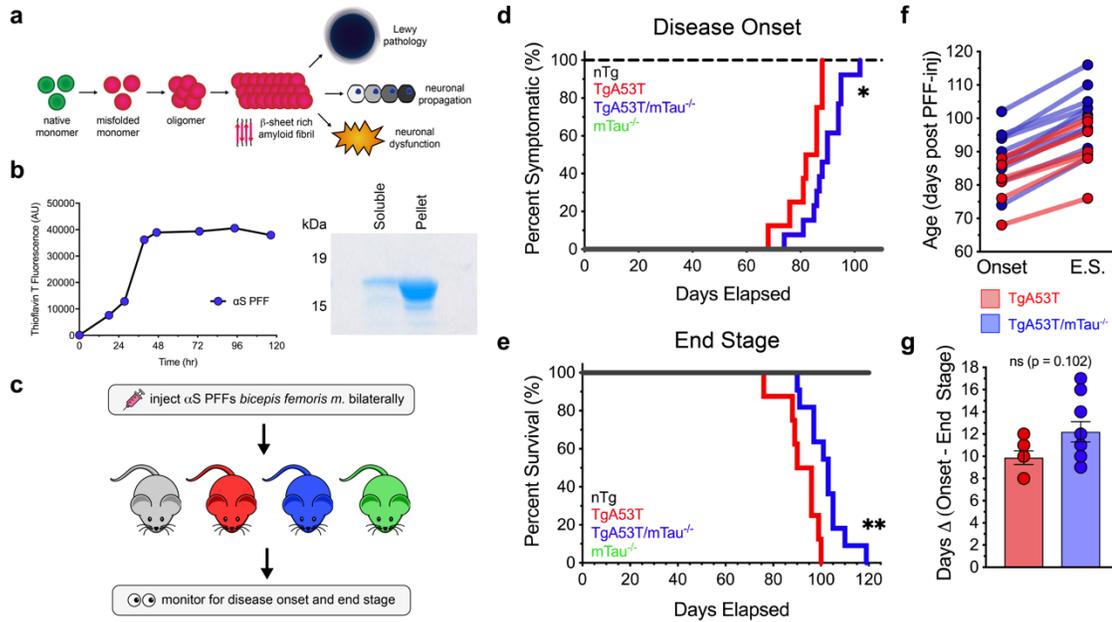


Figure 2.24 – α S^{A53T}-mediated progressive neurological motor deficits and premature lethality are partly tau-dependent. **a.** Model for contributions of amyloid, fibrillar species of α -synuclein (α S) to Parkinson's disease (PD) pathology and pathogenesis. Under physiological conditions, α S exists as a soluble monomeric protein. However, in pathological states, like PD or dementia with Lewy bodies (DLB), α S can misfold and aggregate into higher order species, which likely represent the pathological drivers of PD dementia or DLB. **b.** Left: Monitoring the production and formation of amyloid, fibrillar α S species *in vitro* from monomeric α S via thioflavin T fluorescence (pre-formed fibrils, PFFs). Right: sedimentation assay of α S fibrils. Prepared α S fibrils resolved via SDS-PAGE and stained with Coomassie Brilliant Blue, confirming α S in both the supernatant and pellet. **c.** Overview of experimental design for determining the role of tau in α S PFF-mediated disease onset and end stage (lethality) following bilateral injection of α S PFFs or saline into *biceps femoris muscle* of mice. **d.** Kaplan-Meier curve demonstrating PFF-induced onset of disease (symptomatic). Log-rank (Mantel-Cox) test: $\chi^2 = 9.382$, $df = 1$, $p = 0.0022$. **e.** Kaplan-Meier curve demonstrating PFF-induced end stage (lethality). Log-rank (Mantel-Cox) test: $\chi^2 = 6.288$, $df = 1$, $p = 0.0122$. **f.** Graph depicting the progression of disease, as defined by the days following symptomatic disease onset to reaching pre-defined end stage (E.S.) point. Age refers to days post PFF-injection. Age of onset and age of reaching E.S. of an individual mouse are connected via line. **g.** Quantifying disease progression (days between disease onset and E.S.). Mann-Whitney t test: $U = 21.50$, $p = 0.1025$. $n_{TgA53T} = 8$, $n_{TgA53T/mTau^{-/-}} = 10$. Log-rank and t test: * $p < 0.05$, ** $p < 0.01$. ns: not significant. Error bars represent mean \pm S.E.M. Contributions: HMM.

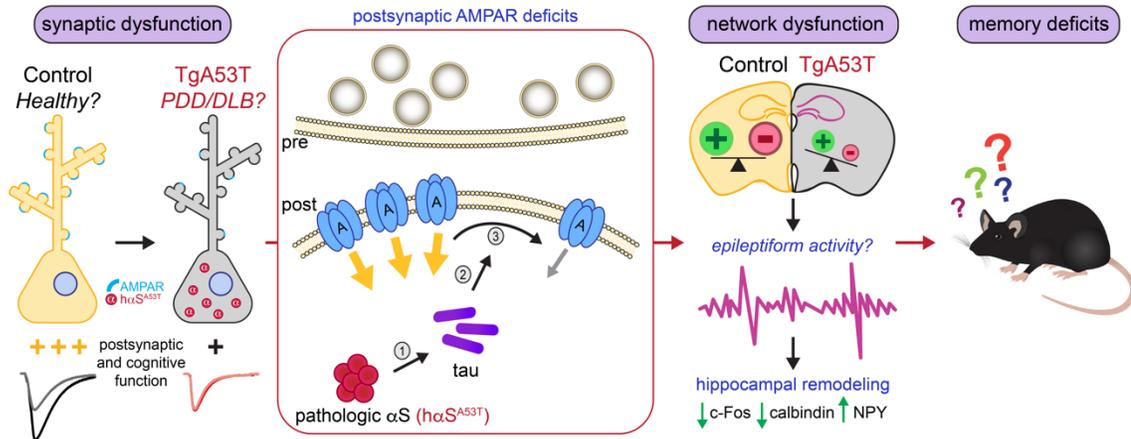


Figure 2.25 – Proposed model for α S-mediated, tau-dependent synaptic and cognitive deficits in $h\alpha S^{A53T}$ mice. Neurons from cognitively-intact nTg control mice display intact AMPA receptor (AMPA) expression and function, and thus display intact synaptic transmission. The presence of pathological species of α S, such as $h\alpha S^{A53T}$ in TgA53T mice leads to progressive postsynaptic synaptic deficits that underlie memory deficits in this model, and potentially PDD or DLB patients, without overt structural alterations at the synaptic or neuronal level. Pathogenic α S ($h\alpha S^{A53T}$) expression or accumulation (1) leads to tau phosphorylation and mislocalization to dendritic spines (2), producing physiological deficits in AMPAR-mediated signaling and synaptic plasticity, likely through reductions in AMPAR expression (3). These $h\alpha S^{A53T}$ -mediated AMPAR deficits culminate in reduced glutamatergic signaling, particularly at hippocampal pyramidal neuron synapses. The AMPAR deficits lead to more global, circuit-level abnormalities in the brains of TgA53T mice. For example, it is possible that $h\alpha S^{A53T}$ -mediated, tau-dependent synaptic depression may also affect inhibitory interneurons, leading to homeostatic imbalance between inhibitory and excitatory neurotransmission and aberrant excitatory activity. These epileptiform changes can then lead to homeostatic responses in hippocampal circuits that attempt to suppress this hyperactivity, representing a potential mechanism underlying the hippocampal remodeling observed in the brains of TgA53T mice. However, future studies are warranted to better elucidate the biochemical, physiological, and structural mechanisms connecting $h\alpha S^{A53T}$ -mediated synaptic dysfunction and aberrant network changes. Ultimately, we hypothesize that $h\alpha S^{A53T}$ -driven, tau-mediated abnormalities at the individual synapse lead to network-level perturbations that together contribute to and exacerbate memory deficits in TgA53T mice. PDD: Parkinson's disease dementia. DLB: dementia with Lewy bodies. Pre: presynaptic neuron. Post: postsynaptic neuron. A: AMPAR. α : $h\alpha S^{A53T}$.

Chapter 3: Pathogenic, fibrillar α -synuclein induces dose-dependent synaptic degeneration and dysfunction

Introduction

The deposition of proteinaceous aggregates in select cell populations is a pathological hallmark shared by many age-related neurodegenerative diseases (Jucker and Walker, 2013). The clinical progression of PD correlates with the appearance of α S inclusions as they spread largely in a stereotypical manner temporally and anatomically throughout the nervous system (Braak and Del Tredici, 2008; Braak et al., 2003), affecting key midbrain and brainstem such as the striatum and substantia nigra, but also other cortical and glutamatergic areas that may precipitate cognitive decline (Kordower et al., 2013; Sulzer and Surmeier, 2013). Importantly, LBs and LNs in the cortex and hippocampus predict memory deficits in Parkinson's disease (Parkinson's disease dementia, PDD) and DLB (Hall et al., 2014; Halliday et al., 2014; Irwin et al., 2012), furthering the relationship between α S abnormalities and α -synucleinopathy disease processes.

Cognitive impairment is a defining symptom of DLB and upwards of 80% of PD patients develop PDD (Irwin et al., 2013). However, the mechanisms driving α S accumulation into LBs and LNs and how other pathological species of α S contribute to the neuronal abnormalities underlying cognitive dysfunction in PDD and DLB remains unknown. In PDD and DLB, the majority of hippocampal Lewy pathology is observed in cell bodies of neurons in the entorhinal cortex and within projections originating from CA2 pyramidal neurons (Adamowicz et al., 2017). However, cognitive dysfunction is best predicted by α S pathology burden in CA1 pyramidal neurons, the output target of CA2 (Adamowicz et al., 2017). From these pathological observations, it can therefore be hypothesized that pathological α S species associating the LNs of CA2 may be released and act trans-synaptically on CA1 postsynaptic sites and cell bodies to mediate synaptic

and cognitive dysfunction. Deficits in synaptic function and hippocampal activity have been observed in neurons acutely exposed to fibrillar species of α S comprised of recombinant h α SST (α S pre-formed fibrils, PFFs) prior to the onset of inclusion formation (Froula et al., 2018; Luna et al., 2018; Volpicelli-Daley et al., 2011; Wu et al., 2019). Acute application of soluble oligomeric species of α S has been shown to also disrupt synaptic plasticity independent of inclusion formation (Diogenes et al., 2012; Ferreira et al., 2015, 2017), supporting this hypothesis and the concept that α S species in PDD and DLB do not need to form Lewy-like inclusions to be neurotoxic.

However, the precise mechanisms mediating these abnormalities remain unknown. As such, there is a need to better define the impact of pathological α S species on neuronal dysfunction prior to the development of overt neuropathology, and the specific pathways involved in order to improve therapies for memory deficits in the α -synucleinopathies. Thus, we asked whether α S PFFs adversely impact synaptic structure and function. In further detail, building upon our recent work that demonstrating that α S-mediated, tau-dependent postsynaptic deficits are critical for memory loss (Teravskis et al., 2018), we propose that memory deficits in both familial and sporadic forms of PDD and DLB may be a product of presynaptic release of toxic α S species, such as those in LNs, to induce postsynaptic abnormalities in a tau-dependent manner.

Upon testing this hypothesis, we show that α S PFFs are capable of inducing tau missorting to postsynaptic structures at a range of α S PFF concentrations in primary hippocampal neurons, leading to deficits in synaptic plasticity. α S PFFs also cause concentration-dependent synaptic loss that is dependent on endogenous α S but not tau, suggesting that different α S PFF-mediated neuronal deficits can occur through distinct mechanisms.

Materials and methods

All animal uses were in accordance with the NIH guidelines for the use of animals in research and approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Experimental group sizes (n) are reported in each figure.

Dissociated neuronal culture electrophysiology

Dissociated neuronal cultures were established from harvested from either Sprague Dawley rats (Charles River; Wilmington, MA) or mouse hippocampi postnatal day 0 or 1 (P0-P1) as previously described (Teravskis et al., 2018) with modifications. Briefly, mouse hippocampi were dissected out and stored individually by pup in Hibernate A media (Brain Bits; Springfield, IL) at 4°C. After dissection, hippocampi were dissociated, and plated on onto 35 mm μ -dishes containing a poly-D-lysine- (Sigma-Aldrich; St. Louis, MO) and laminin-coated (Sigma-Aldrich) polymer coverslip (Ibidi; Fitchburg, WI). 24 hours (hrs) after initial plating, media was removed and NbActiv4 growth media was added (Brain Bits). NbActiv4 media was refreshed every 3-4 days by removing 1 mL media in the dishes and adding 1 mL fresh media. For all procedures and media exchanges, solutions were equilibrated in a tissue culture incubator (37°C, 5% CO₂) for at least 2 hrs prior to use. The age of *in vitro* dissociated cultured hippocampal neurons began with the day of initial plating, and each day that followed was counted as one day *in vitro* (DIV). All experiments were performed on neurons from at least 3 independent cultures with a minimum of 5 animals per culture. Miniature excitatory postsynaptic currents (mEPSC) were recorded from cultured dissociated mouse hippocampal neurons at 21–25 DIV with a glass pipette (resistance of ~5 M Ω) as previously described (Teravskis et al., 2018). Recordings ranged from 3-15 minutes (min) and stable traces longer than 1 min in duration were analyzed. All mEPSCs >2 pA were manually counted with MiniAnalysis (Synptosoft Inc; Fort Lee,

NJ). Each mEPSC event was visually inspected and only events with a distinctly fast-rising phase and a slow-decaying phase were accepted. Relative cumulative frequencies were derived from individual events and the averaged parameters from each neuron were treated as single samples in any further statistical analyses.

***In vitro* neuronal imaging and analysis**

At 6-8 DIV, primary hippocampal neurons plated on 35 mm dishes underwent calcium-phosphate transfection (Jiang and Chen, 2006) with modifications. Neurons were incubated with the DNA-calcium-phosphate precipitate solution for 3 hrs in a standard tissue culture incubator (37°C, 5% CO₂). After this incubation period, media containing the transfection precipitate was removed and replaced with fresh, equilibrated NbActiv4 media. After transfection, NbActiv4 media was refreshed every 3-4 days by removing 1 mL media in the dishes and adding 1 mL fresh media until beginning experiments at 21 DIV, a time at which neurons consistently express high numbers of mature dendritic spines *in vitro* (Hoover et al., 2010).

For live cell imaging, 35 mm cell culture dishes were fitted a custom holding chamber on a fixed platform above an inverted Nikon Eclipse TE300 microscope (Nikon; Melville, NY) placed on an X-Y translation stage (Burleigh; Thorlabs Inc, Newton, NJ). Images were subsequently acquired at 60x magnification in a Z-stack comprised of 15 images at 0.5 μm intervals. Images were both acquired and analyzed using MetaMorph 7.1.0 (Molecular Devices; San Jose, CA). Live image stacks were then processed via 2D deconvolution and averaged into a single, stacked image. Dendritic spines were characterized as protrusions with a head width more than 50% the width of the neck. Spines were manually counted and then normalized to dendritic length within and between neurons. Imaging and electrophysiological experiments were performed from at least 2 independent cultures.

Immunohistochemical analyses

For immunohistochemical studies, neurons were plated on poly-D-lysine- (Sigma-Aldrich) and laminin-coated (Sigma-Aldrich) glass coverslips in 6-well plates. After α S PFF treatment, neurons were fixed with 4% paraformaldehyde (PFA). Prior to staining neurons were washed in TBS, permeabilized with 0.1% Triton X-100 in tris-buffered saline (TBS) and blocked in 50% Background Sniper solution in TBS (Biocare Medical; Pacheco, CA). Primary and secondary antibodies were diluted to target concentrations in 5% Background Sniper in TBS with 0.1% Tween 20 (TBSTw). Primary antibody incubation was conducted overnight at 4°C and secondary antibody incubation was for 1 hr at room temperature in the dark. Alexa Fluor Plus (Thermo Fisher Scientific) secondary antibodies were used as dictated by primary antibody host. If required, sections were then 4',6-diamidino-2-phenylindole (DAPI)-counterstained (Thermo Fisher Scientific; Waltham, MA). All coverslips were then mounted on slides with ProLong Gold antifade mounting reagent (Thermo Fisher Scientific) for future imaging. Confocal microscopy images were acquired with the Nikon C2 Confocal Microscope System (Nikon; Melville, NY). Imaging processing and analysis were performed using NIS-Elements software (Nikon). Staining, imaging, and analysis were performed in collaboration with the University of Minnesota's University Imaging Centers on the Twin Cities campus.

Preparation of human wild-type α S monomers and pre-formed fibrils ($h\alpha$ S^{WT} PFFs)

A schematic is outlined in **Figure 3.1**. In detail: recombinant full length human wild-type α S monomers and pre-formed fibrils ($h\alpha$ S^{WT} PFFs) were generated following the protocol established by Volpicelli-Daley et al., 2014. α S monomers destined for experiments were aliquoted and stored at -80°C at 5 mg/mL. In preparation for use,

monomers were thawed on ice, diluted to a working concentration of 0.25 mg/mL (250 μ g/mL) in phosphate-buffered saline (PBS), and then spun down for 20 mins at 100,000 g (30 PSI) via Beckman Airfuge[®] CLS air-driven ultracentrifuge (Beckman Coulter; Indianapolis, IN) to pellet any aggregates that may have formed. The resulting supernatant from this spin was then used for downstream experiments as monomeric α S.

α S PFFs were generated by first incubating purified recombinant monomeric α S at 5 mg/mL in PBS at 37°C with continuous shaking for 7 days via ThermoMixer (Eppendorf; Hamburg, Germany). During this shaking process, the presence of amyloid α S fibrils production was assessed and confirmed using Thioflavin T fluorometry. Following shaking and saturation of Thioflavin T fluorescence levels, α S PFFs were subsequently aliquoted and stored at -80°C. The generation of α S PFFs and their seeding capacity were confirmed through sedimentation assay, subjecting an aliquot of α S PFFs to ultracentrifugation, and assessing the amount of α S in pellet and supernatant via SDS-PAGE (BioRad) and Coomassie Brilliant Blue (Thermo Fisher Scientific) staining. For peripheral injections into mice, 5 mg/mL α S PFF aliquots in PBS were taken from -80°C, thawed at room temperature, and diluted to a working concentration of 0.25 mg/mL (250 μ g/mL) in PBS. α S PFFs at this concentration were then sonicated utilizing a Fisher Scientific Branson micro probe tip sonicator (Fischer Scientific; Hampton, NH) with 60 pulses at 20% power for a total of 120 sec, 1 sec “on”, 1 sec “off”. Following sonication, PFFs were ready for further dilution and application to cells.

Plasmid constructs

Both eGFP-tau and DsRed plasmids were expressed in the pRK5 vector and driven by the cytomegalovirus (CMV) promoter. The eGFP-tau construct (#46904, Addgene; Watertown, MA) expresses wild-type human four-repeat tau lacking the N-terminal repeat

domain (4R0N) and contained exons 1, 4, 5, 7, 9-14, and intron 13. The DsRed plasmid (Clonetech; Mountain View, CA) was cloned into the pRK5 construct.

Antibodies

A detailed list of all antibodies used for experiments and studies here can be found in **Table 2.1**.

Experimental design and statistical analyses

All statistical analyses were performed in Prism 8.1.0 (GraphPad Software; San Diego, CA). Data visualization and presentation were performed via Prism (GraphPad). For parametric data: one- and two-way ANOVAs were utilized for one and two variable analysis on multiple groups, respectively; t tests were also performed when analyzing only two groups. For nonparametric data: Kruskal-Wallis one-way ANOVA for three or more groups; Mann-Whitney test for two groups. Posthoc analyses were performed on all data that were significantly different. Welch's correction, Sidak's posthoc, and Tukey's posthoc analyses were used due to considerations for within-group variances and sample size. For all, statistical significance was set for $\alpha = 0.05$. Data representations are described in figure legends.

Results

α S pre-formed fibrils contribute to synaptic dysfunction and synapse loss in a dose-dependent manner

We first hypothesized that 1) α S PFF-mediated synaptic abnormalities are drivers of memory deficits in PDD and DLB, and 2) may occur through tau missorting to dendritic spines, a phenomenon associated with postsynaptic dysfunction and cognitive decline in models of AD (Hoover et al., 2010; Miller et al., 2014). To these initial hypotheses, postnatal rat primary hippocampal neurons cultures were established, co-transfected with DsRed to visualize neuronal structures and eGFP-tagged WT human tau (eGFP-tau) plasmids, and aged to 21 days *in vitro* (DIV), at which mature spines are present. At 21 DIV, neurons were treated with α S monomers or PFFs and imaged 24 hours (hrs) post-treatment (**Figure 3.2a**). While neither saline nor monomeric α S species contributed to the mislocalization of tau, acute exposure of primary hippocampal neurons to α S FFs at both low (0.10 μ g/mL) and high (4.00 μ g/mL) concentrations was sufficient to induce tau missorting to dendritic spines (**Figure 3.2a-b**). However, α S PFF treatment at high doses (4.00 μ g/mL) also produced a loss of spines at this 24 hrs time point, as indicated by a reduced spine density (spine/pixel) count along dendrites (**Figure 3.2a and c**). Notably, this α S PFF-induced spine collapse was absent in neurons exposed to lower α S PFF concentrations (0.10 μ g/mL), indicating a divergence between tau missorting to dendritic spines and loss of dendritic spines

To further investigate the concentration-dependent synaptotoxic effects of α S PFFs, primary hippocampal neurons were fixed 24 hrs post- α S PFF treatment and subsequently stained for pre and postsynaptic markers, Synapsin 1/2 and PSD95, respectively. Although α S PFFs at 4.00 μ g/mL begin to induce neuron death beginning around 14 days post-treatment in culture (Volpicelli-Daley et al., 2014), qualitative analysis via confocal

microscopy suggest a reduction in colocalization of Synapsin 1/2 and PSD95, which may be indicative of synapse loss (**Figure 3.3**). As such, synaptic degeneration may represent early, acute responses to α S PFFs that precede overt neuropathology and neurodegeneration. Along these lines, both oligomeric and fibrillar forms of α S have been demonstrated to induce neuronal loss both *in vitro* and *in vivo*, suggesting that, with time, neuronal viability is compromised in the presence of pathogenic multimeric α S species (Luk et al., 2012a; Peelaerts et al., 2015; Volpicelli-Daley et al., 2011; Winner et al., 2011).

Tau missorting to dendritic spines is associated with electrophysiological deficits in glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPA) signaling (Hoover et al., 2010; Teravskis et al., 2018). α S PFFs have also been reported to induce functional neuronal deficits *in vitro* from a network perspective as suggested by calcium imaging (Volpicelli-Daley et al., 2011). We therefore next asked if α S PFF-induced tau mislocalization was also associated with functional deficits *in vitro*. At 21 DIV, rat primary hippocampal neurons were treated with low (0.10 μ g/mL) or high (4.00 μ g/mL) concentrations and analyzed 24 hrs following via whole cell patch clamp electrophysiology. Specifically, mini excitatory postsynaptic currents (mEPSC) were recorded and analyzed for frequency and amplitude (**Figure 3.4**). Acute exposure to α S PFFs at both high and low concentrations were sufficient to reduce mEPSC frequency, a metric of excitatory presynaptic vesicle release (**Figure 3.4a and c-d**). mEPSC amplitude, a measure of postsynaptic AMPAR presence and function, was also significantly depressed in neuron treated with α S PFFs (**Figure 3.4b-d**), suggesting that α S PFF-induced postsynaptic AMPAR dysfunction may indeed be a consequence of tau missorting to dendritic spines. Taken together, these electrophysiological findings suggest that α S PFFs are capable of altering synaptic function and structure, reflected in the depressed mEPSC frequencies and amplitudes in neurons acutely exposed to high or low

concentrations of α S PFFs. The reduced mEPSC frequency observed in neurons acutely exposed to 0.10 μ g/mL or 4.00 μ g/ml α S PFFs may be multi-factorial. High dose α S PFF-induced synapse loss can present as reduced mEPSC frequency due to less opportunities for presynaptic vesicle release. However, increased α S expression is also associated with reduced vesicle release (Nemani et al., 2010), and thus may represent a presynaptic consequence of α S PFF exposure and internalization.

α S PFF-induced tau missorting and spine loss involve different mechanisms

To investigate the mechanisms underlying α S PFF-induced tau missorting and synaptic deficits in primary hippocampal neuronal cultures, we next asked if specific posttranslational modifications of tau were required. In particular, tau phosphorylation and GSK3 β activity, an established kinase of tau, are critical components for the induction of tau mislocalization into spines (Hoover et al., 2010; Teravskis et al., 2018). Consistent with these previous studies, pharmacological inhibition of GSK3 β via 500 nM CHIR 99021 (Tocris; BioTechne, Minneapolis, MN) prior to α S PFF exposure is sufficient to block tau missorting into dendritic spines at both low (0.10 μ g/mL) and high (4.00 μ g/mL) concentrations (**Figure 3.5a-b**). Surprisingly, α S PFF-induced spine loss at high concentrations was also rescued as neurons pre-treated with CHIR 99021 for 12 hrs prior to 4.00 μ g/mL α S PFF exposure for 24 hrs displayed similar spine density levels as compared to 0.10 μ g/mL α S PFF and control conditions (**Figure 3.5a and c**). Although tau missorting and associated AMPAR deficits are not thought to be connected with spine collapse (Hoover et al., 2010; Miller et al., 2014; Teravskis et al., 2018), it is possible that α S PFFs have other pathological actions downstream of GSK3 β that ultimately produce the synaptotoxic effects observed here in culture.

α S PFF-induced tau missorting is a pathological process that likely requires internalization of fibrils into neurons. However, it is also possible that extracellular α S PFFs contribute to neuronal abnormalities and degeneration. Cellular prion protein (PrP^C) at the surface of postsynaptic densities is implicated as a mediator of amyloid- β (A β)-induced synaptic dysfunction in primary neurons exposed to A β oligomers and memory deficits in mouse models of Alzheimer's disease (AD) (Gimbel et al., 2010; Laurén et al., 2009; Um et al., 2012, 2013). More recent studies have found that α S oligomer-mediated synaptic impairment occurs in a PrP^C-dependent manner (Ferreira et al., 2017). Therefore, we next asked if the pathologic changes following α S PFF exposure could be attributed through extracellular effects of fibrils, specifically via PrP^C. Neurons pre-treated with 100 nM 6D11 (BioLegend; San Diego, CA), an antibody against PrP^C, did not block α S PFF-induced tau missorting (**Figure 3.6a-b**). However, this antibody-mediated blocking of PrP^C signaling prevented spine loss in primary hippocampal neurons exposed to high concentrations (4.00 μ g/mL) of α S PFFs (**Figure 3.6a and c**). Taken together, it is evident that α S PFFs exert a range of neurotoxic effects through distinct intracellular and extracellular mechanisms. However, to further clarify these mechanisms, important future experiments are essential, which include controlling for key manipulations here, such as CHIR in dimethyl sulfoxide (DMSO), and immunoglobulin G (IgG) for the 6D11 IgG monoclonal antibody.

Endogenous α S has differential roles on α S PFF-induced abnormalities in primary neurons

Endogenous mouse α S is a critical determinant of α S PFF-mediated neuronal changes, including aggregate formation within neurons, spine dynamics, and disruption of neurotransmission (Froula et al., 2018; Volpicelli-Daley et al., 2011). We therefore next

asked if tau mislocalization to dendritic spines and the dose-dependent effects on these postsynaptic structures required endogenous α S. To accomplish this, primary hippocampal neurons from *SNCA*^{-/-} mouse pups were cultured, co-transfected with DsRed and eGFP-tau, treated with α S PFFs, and live imaged consistent with the previous experiments. α S PFF-mediated tau missorting at low and high concentrations was unaffected by removal of endogenous α S expression (**Figure 3.7a-b**). However, spine loss associated with exposure to high concentrations of α S PFF (4.00 μ g/mL) was absent in *SNCA*^{-/-} neurons (**Figure 3.7a and c**), further highlighting the differential pathways mediating α S PFF-induced neuronal dysfunction. *SNCA*^{-/-} litters were obtained from homozygous *SNCA*^{-/-} parents, precluding the ability to compare the role of endogenous α S between littermates. As such, while studies using culture neurons from C57BL6/J (BL6) hippocampi were not performed in parallel, tau missorting and synapse loss with high dose α S PFFs has been observed in multiple experiments. Tau mislocalization may be a primary effect of α S PFFs in cultured neurons. However, given these findings in *SNCA*^{-/-} neurons, α S PFF-induced spine collapse likely requires corruption and recruitment of endogenous α S to specifically mediate these toxic effects on postsynaptic structures.

Discussion

Despite the central role of α S in PDD and DLB pathogenesis, the manners in which different pathological species of α S contribute to neuronal dysfunction and degeneration remain unknown. Determining the driving forces behind α S aggregation, which species of α S are pathogenic and transmissible, and their mechanisms, are critical for the advancing our understanding of hippocampal dysfunction and memory deficits in the α -synucleinopathies. In this study we investigated the structural and functional changes

associated with acute exposure to α S PFFs in primary hippocampal neuronal cultures, motivated by our hypothesis that α S PFFs may represent a pathological species that contributes to the neuronal dysfunction underlying cognitive impairment in PDD and DLB.

To date, monitoring α S-rich Lewy pathology in the cortex and limbic system represents one of the most sensitive and specific pathological measures for dementia in PD (Ballard et al., 2006; Halliday et al., 2014). However, it remains unknown if this pathology is a primary driver of neuronal abnormalities leading to memory changes or if this pathology merely reflects underlying disease processes. Neuronal dysfunction in cholinergic hippocampal neurons and CA1 pyramidal neurons, as opposed to neuropathology or neurodegeneration, are associated with impaired memory performance and dementia in PD and DLB (Adamowicz et al., 2017; Hall et al., 2014). To better evaluate connection between pathological species of α S and cellular deficits, we utilized a seeded model of α -synucleinopathy that recapitulates key aspects of disease pathogenesis and progression (Volpicelli-Daley et al., 2014). Using this approach, acute application of α S PFFs to primary hippocampal neuronal cultures was able to induce pathological tau mislocalization to dendritic spines by 24 hrs post-treatment. However, we also observed concentration-dependent synaptic alterations in α S PFF-treated neurons. While low (0.10 μ g/mL) and high (4.00 μ g/mL) amounts of α S PFFs were both capable of inducing tau missorting, neurons exposed to high concentrations of α S PFFs also displayed a reduced spine density when imaged 24 hrs post treatment. Correspondingly, we found that this 24 hr α S PFF exposure time frame was sufficient to induce deficits in spontaneous synaptic neurotransmission as measured by reductions in mEPSC amplitude as well as mEPSC frequency. Consistent with previous studies (Hoover et al., 2010; Miller et al., 2014), α S PFF-induced tau missorting also requires GSK3 β , a known tau kinase. Interestingly, α S PFF-induced spine loss was rescued via pharmacological GSK3 β inhibition, pointing to a

larger involvement of this kinase in α S PFF-mediated neurodegeneration. While α S PFFs are capable of endocytosis (Volpicelli-Daley et al., 2011), their extracellular effects cannot be excluded. Inhibition of PrP^C was not sufficient to block tau missorting but did rescue α S PFF-dependent spine collapse, suggesting that pathogenic α S species exert their neurotoxic properties via differing pathways. Endogenous α S, known to aggregate and mediate neuronal deficits in response to α S PFF exposure, was also required for α S PFF-mediated synaptotoxicity but not tau missorting. Taken together, our findings demonstrate a dose-dependent effect of α S PFFs on primary hippocampal neurons in culture, along with the initial efforts to understand the mechanisms by which α S PFFs contribute to disease pathogenesis and the role for α S aggregates and Lewy pathology in these processes.

α S is an abundant presynaptic protein enriched, comprising upwards of 1% of all cytosolic proteins, yet resists aggregation under physiologic conditions (Stefanis, 2012). The advent of the α S fibril model for studying PD pathogenesis is unique in its capacity to induce pathology and inclusion formation in cells that express endogenous levels of α S (Luk et al., 2012a; Volpicelli-Daley et al., 2011, 2014). However, the induction of endogenous α S aggregate formation begins with application of supra-physiologic levels of recombinant α S in the form of PFFs. Up to this point, the concentration-dependent effects of oligomeric or fibrillar α S on cultured neurons had not been examined, putting the relevance of the findings in question with respect to disease and translational relevance (Ferreira et al., 2017; Froula et al., 2018; Luna et al., 2018; Volpicelli-Daley et al., 2011). Thus, in addition to examining structural and functional synaptic changes associated with acute α S PFF exposure in primary hippocampal neurons at doses, these studies previously utilized, 4.00 μ g/mL (~300 nM), this thesis work also examined a lower concentration, 0.10 μ g/mL (~8 nM), hypothesizing that if α S PFFs indeed represent a

transmissible species responsible for PDD and DLB pathogenesis, the amounts found in the extracellular milieu following excretion or leakage from diseased neurons are likely to be much lower. This was partially validated as a concentration 0.10 $\mu\text{g}/\text{mL}$ of αS PFFs was effective at inducing pathologic tau missorting to dendritic spines and synaptic deficits as measured via reduced frequency and amplitude of mEPSCs. While the established 4.00 $\mu\text{g}/\text{mL}$ concentration produced similar effects, it also resulted in spine and synapse loss. Although synapse loss and hippocampal atrophy can be observed in PDD and DLB (Camicioli et al., 2003), the lack of prominent neurodegenerative features in the hippocampus is more commonly a distinguishing feature for the diagnosis of PDD or DLB as compared to other dementias such as AD (Halliday et al., 2014; McKeith et al., 2017), again suggesting to a mechanism underlying memory deficits that is more centered on synaptic dysfunction as opposed to overt synapse loss.

An interesting finding of our studies is the discrepancy between synapse loss and neurophysiological dysfunction at 0.10 and 4.00 $\mu\text{g}/\text{mL}$ of αS PFFs. Both doses induce tau missorting to dendritic spines and correspondingly AMPAR deficits as measured via reductions in mEPSC amplitude. Depressed mEPSC frequency is observed at both concentrations tested, but it is possible that origins of these observations may differ. As 4.00 $\mu\text{g}/\text{mL}$ αS PFF concentrations are associated with spine and synapse loss, mEPSC frequency reduction in this setting may be confounded by overt synapse loss. However, consistent with its role in modulation of presynaptic neurotransmission and vesicle dynamics, αS overexpression is associated with reduced presynaptic vesicle in the absence of synapse loss (Larsen et al., 2006; Nemani et al., 2010; Teravskis et al., 2018). As such, this represents the likely mechanism for mEPSC deficits in neurons exposed to 0.10 $\mu\text{g}/\text{mL}$ αS PFFs, consistent with previous studies establishing αS PFF internalization and trafficking to presynaptic terminals, the site of αS PFF-induced pathology initiation

(Karpowicz et al., 2017; Volpicelli-Daley et al., 2011). It will be important to further examine spine and synapse loss following acute α S PFF exposure to determine if these changes are transient or irreversible responses to toxic levels of pathogenic α S. α S overexpression is also known to reduce synapsin levels (Larson et al., 2017; Nemani et al., 2010), and thus the qualitative reduction we observe in synapsin and PSD95 colocalization may instead reflect this change as opposed to “true” reductions in presynaptic terminals.

In addition to the spine loss observed here, 4.00 μ g/mL of α S PFFs induces altered spine dynamics, and synaptic deficits on the order of hours post-exposure (Froula et al., 2018; Wu et al., 2019), followed by neuronal loss beginning around 14 days post-exposure (Volpicelli-Daley et al., 2011, 2014). To that end, the use of lower α S PFF doses may provide for more disease-relevant studies and conclusions, particularly with respect to the dissection between α S-mediated neuronal deficits and α S inclusion formation. Increased endogenous α S expression within neurons is associated with faster induction of α S pathology and cell death (Luna et al., 2018). As such, it is possible that some of the phenotypes observed in neurons treated with 4.00 μ g/mL may be due to their capacity to induce proteostatic, lysosomal, and mitochondrial stress at these higher concentrations (Dryanovski et al., 2013; Karpowicz et al., 2017), a potential byproduct of overwhelming cells with exogenous α S rather than a more physiologic, disease-relevant process that could be assumed from leakage of pathologic α S from presynaptic terminals (Vasili et al., 2019).

The ability for α S PFFs to compromise synaptic and neuronal function has been previously observed (Di Scala et al., 2016; Dryanovski et al., 2013; Froula et al., 2018; Luna et al., 2018; Volpicelli-Daley et al., 2011), but only examined at time points that would concomitant with the presence of α S inclusions, precluding the capacity to determine what pathological forms of α S are directly responsible for the changes observed. Moreover, the

mechanisms underlying these changes and concentration-dependent nature of these changes was not known as all studies employed a single concentration that is now known to greatly exceed the physiologic concentration of endogenous α S and what is needed to seed aggregate formation (Afitska et al., 2019; Wilhelm et al., 2014). Here, we demonstrate that low doses of α S PFFs known to favor aggregate formation are capable of inducing synaptic deficits without the rapid neurotoxic effects associated with higher doses. In addition to these dose-dependent phenotypes, albeit at extreme ends of the concentration spectrums, we begin to establish the mechanisms underlying α S PFF-mediated neuronal dysfunction, proposing that extracellular and intracellular pools of α S PFFs together contribute to disease pathogenesis.

Recent work demonstrating that the concentration of α S at the synapse is around 20 μ M (Wilhelm et al., 2014), a 30-fold difference from the established 4.00 μ g/mL α S PFF dosage, further supports the pursuit of dissecting out α S PFF-induced dose-dependent versus dose-independent neuronal abnormalities. The ability of this high dose to corrupt and recruit endogenous α S into fibrillar inclusions may indeed represent a relevant disease process for transmission within the nervous system, but it may also represent an artifact of elevated intraneuronal α S. Lower α S concentrations favor the monomeric state of α S, and recent work has demonstrated that a concentration of 3-5 μ M of α S PFFs represents a favorable concentration fibril seeds to drive recruit monomeric α S into *de novo* fibrils (Afitska et al., 2019). The 0.10 μ g/mL dosage corresponds to approximately 8 nM and thus it is possible that this concentration will also seed monomeric α S conversion to fibrils, although on a longer time scale as compared to the 4.00 μ g/mL dose. To that end, prolonging the lag time for inclusion formation will provide for further dissection of neuronal abnormalities due to soluble or pre-fibrillar α S aggregates and those due to fibrillar α S inclusions. Moreover, it provides for the observation that tau missorting synaptic

dysfunction observed in neurons treated with 0.10 $\mu\text{g}/\text{mL}$ αS PFFs is less likely a product of αS aggregation but the intrinsic pathogenicity of fibrils within neurons through varying mechanisms that can be revealed through future studies.

While the mechanisms describing how these pathological αS species corrupt endogenous αS remains unknown, it is clear that distinct conformational strains of fibrillar αS are capable inducing distinct neuropathological phenotypes (Guo et al., 2013; Peelaerts et al., 2015). Additionally, the rapid formation of αS inclusions and neurodegeneration observed in α -synucleinopathy animal models following αS PFF injection (Luk et al., 2012b; Masuda-Suzukake et al., 2013) may not just be a consequence of overt αS overexpression but also species of αS expressed. Cross-species seeding is clearly achieved at high doses of αS PFFs both *in vitro* and *in vivo* (Luk et al., 2012b; Volpicelli-Daley et al., 2011). However, exogenously human αS fibrils display enhanced pathogenicity when introduced to neurons expressing human αS expression as compared to mouse αS (Luk et al., 2016). It is therefore reasonable to conclude that the αS PFF-induced spine collapse is distinct from αS PFF-induced tau missorting. Here, we show that spine loss, but not tau missorting, requires both endogenous mouse αS expression as well as PrP^C signaling. One possible explanation for this finding follows the host compatibility studies in αS . Bigenic mice overexpressing human αS and tau display increased fibrillization of each protein as compared to singly transgenic mice, pointing to a synergistic interaction between these proteins from an aggregation perspective (Giasson et al., 2003). This concept has been furthered via the αS PFF model in primary neurons overexpressing human tau as αS PFFs are able to induce cross-seeding of tau aggregation in these cells, but not those cultured from non-transgenic mice (Guo et al., 2013). The overexpression of human tau in our studies parallels these findings. For

example, human α S PFFs can directly influence certain pathologic processes, such as tau missorting, likely due to the host species matching, whereas other aspects of α S PFF-induced neurodegeneration, such as spine and synapse loss, require protein intermediates like GSK3 β and PrP^C, as well as endogenous α S, to mediate these toxic effects. This is consistent with the concept that pathogenic A β and α S oligomeric species can act through distinct intracellular and extracellular mechanisms that ultimately converge to comprise synaptic plasticity and neuronal function (Ferreira et al., 2017; Laurén et al., 2009; Miller et al., 2014; Um et al., 2012).

Prior studies support a connection between the burden of α S neuropathology and eventual neurotoxicity in α S PFF models (Luna et al., 2018; Masuda-Suzukake et al., 2014; Volpicelli-Daley et al., 2011). However, neither neuropathology nor neurodegeneration are necessary for the manifestation of α S-mediated synaptic deficits *in vitro* and *ex vivo* (Ferreira et al., 2017; Teravskis et al., 2018), highlighting a divergence between aggregation and specific neuronal abnormalities. This relationship is supported by the findings here that α S PFFs alter neurotransmission at time points known to precede α S PFF-induced development of Lewy-like pathology. Significantly, α S-mediated neuronal dysfunction, independent of α S inclusion formation or neuronal loss, is sufficient to precipitate memory deficits in TgWT and TgA53T α -synucleinopathy mouse models (Ferreira et al., 2017; Magen et al., 2012; Teravskis et al., 2018). This concept is central to the studies presented in Chapter 2 of this thesis as h α S^{A53T}-mediated synaptic and memory deficits occur without the need for loss of synapses or neurons within the hippocampus. Furthermore, while these deficits require tau, they do not require alterations in α S expression or levels of pathological oligomeric, fibrillar, or insoluble species. It is possible, however, that pathogenic aggregates in TgA53T hippocampal neurons are essential for disease pathophysiology but may be upstream of tau in the of α S-mediated,

tau-dependent, synaptic and memory deficit pathway. This suggests that, in the context of low dose α S PFFs (e.g. 0.10 μ g/mL), synaptic abnormalities are likely to be a direct consequence of fibrillar α S species, independent of inclusion formation or other neurodegenerative changes associated with higher α S PFF doses, synapse loss in particular.

These observations hold clinical relevance in several capacities. First, although increased cortical and hippocampal Lewy pathology burden is a sensitive and specific measure for predicting cognitive impairment, it is not observed in all patients with a diagnosis of PDD or DLB (Colosimo et al., 2003; Halliday et al., 2014). In addition, hippocampal neurodegeneration is not a common finding in PDD and DLB, with the observation of preserved hippocampal volumes successfully distinguishing dementia in PDD and DLB from other causes like AD (Hely et al., 2008; Kantarci et al., 2016; McKeith et al., 2017). Lastly, hippocampal neuronal dysfunction in specific regions such as CA1 correlates better with impaired memory performance than areas with a higher α S pathology burden such as CA2, the entorhinal cortex, and parahippocampal gyri (Adamowicz et al., 2017; Hall et al., 2014; Harding and Halliday, 2001). However, as several of these regions project onto CA1 pyramidal neurons, it can be hypothesized that α S-induced memory deficits are a consequence of hippocampal synapse and circuit dysfunction rather than structural or neuronal degeneration. For example, neurons positive for Lewy-pathology (LNs in particular) that synapse onto CA1 pyramidal neurons may transmit pathological α S species, such as fibrillar α S, in a trans-synaptic manner onto their CA1 targets to mediate synaptic dysfunction in CA1 neurons independent of Lewy-pathology development.

Tau pathology is gaining relevance as a biomarker for cognitive dysfunction in PDD and DLB (Coughlin et al., 2018; Irwin and Hurtig, 2018; Irwin et al., 2013), but how tau

contributes to α S-driven cognitive dysfunction is not fully established. These findings support a model that tau plays an active role in mediating synaptic dysfunction and AMPAR deficits underlying memory deficits in PDD and DLB, demonstrating that α S PFF introduction leads to impairments in neurotransmission at both pre and postsynaptic compartments, partially mediated via tau missorting to dendritic spines, prior to the onset of Lewy-like pathology (summarized in **Figure 3.8**). Collectively, these findings provide insight into how pathogenic species of α S are likely to mediate cognitive decline in the α -synucleinopathies, helping to improve disease models and the potential translational relevance for these studies and future therapeutic approaches aimed at addressing memory deficits in PDD and DLB.

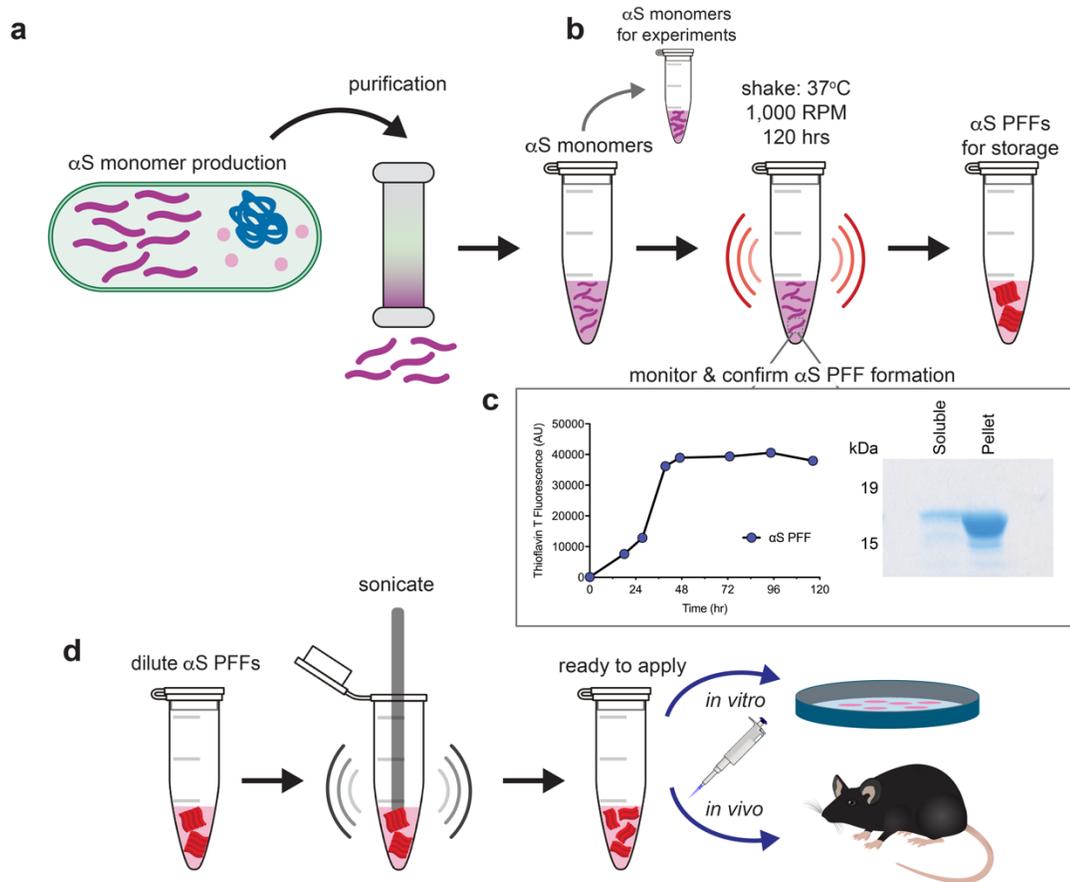


Figure 3.1 – Schematic depicting the protocol for generating and using recombinant α S monomeric and pre-formed fibril (PFF) species. **a.** Generation of α S PFFs begins via production of α S monomers via bacterial cell expression system, classically *E. coli*. Bacterial production of α S is followed by cell lysis, ammonium sulfate precipitation of proteins, and isolation of monomeric α S via anion exchange and size exclusion chromatographic processes. **b.** Monomeric α S at this point can be stored for future experimental application or used to generate PFFs via continuous shaking at set conditions for up to 120 hrs (5 days). **c.** Production of α S PFFs from monomers is monitored via Thioflavin T (ThioT) fluorescence assays. After ThioT fluorescence intensity values have plateaued, PFF production is considered complete. Performing sedimentation assay via centrifugation of PFFs to produce supernatant and pellet fractions that are then resolved via SDS-PAGE is also essential to confirm α S PFF production and levels in both fractions. **d.** After production, confirmation, and storage, it is critical to dilute and sonicate α S PFFs prior to use to break up larger aggregates and generate a majority of PFFs that are short, 50 nm or less in length, as these have proven to be the most effective in seeding pathology and likely other precipitating neurotoxic effects. Contributions: HMM.

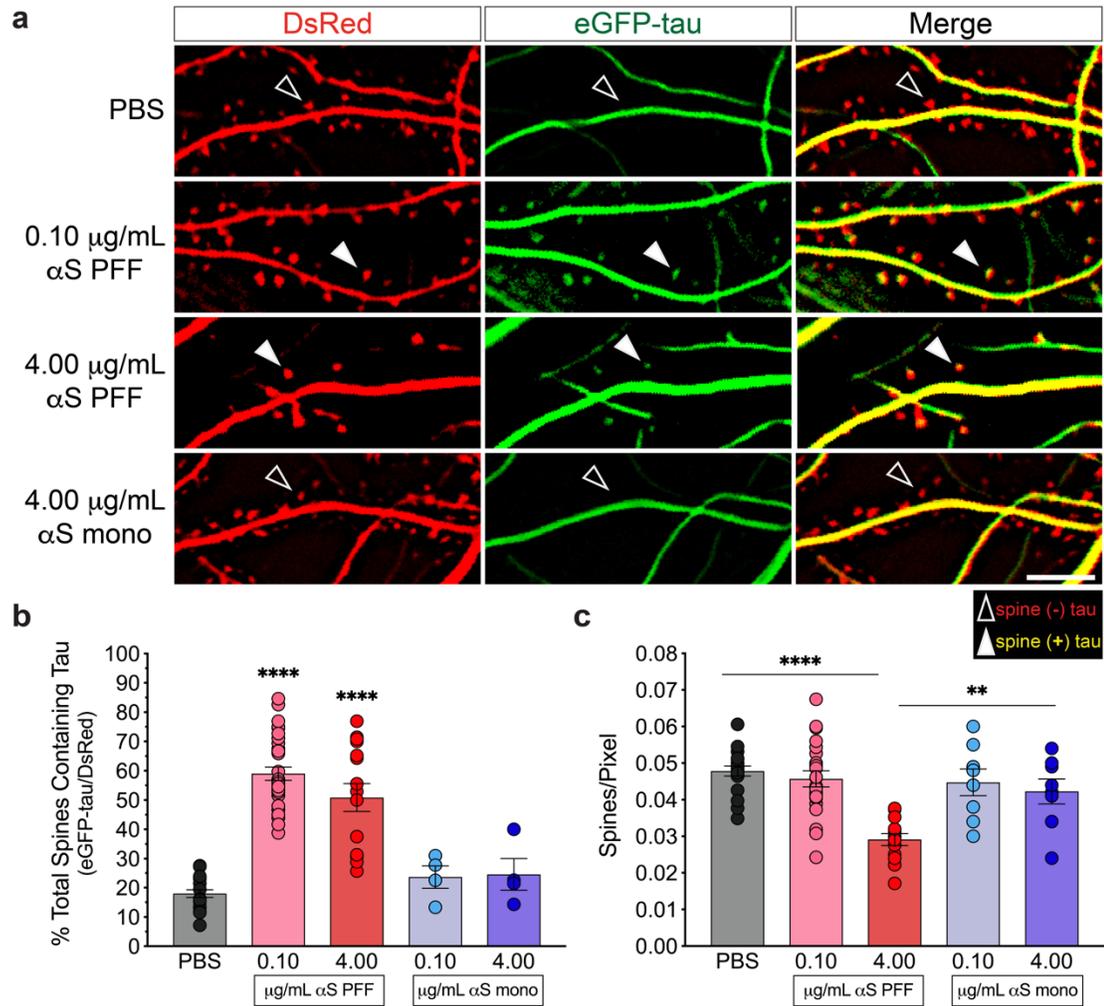


Figure 3.2 – α S pre-formed fibrils induce tau-mislocalization to dendritic spines and dose-dependent spine loss. **a**. Cultured rat primary hippocampal neurons were co-transfected with plasmid expressing DsRed to visualize neuronal architecture or eGFP-tagged to 4R0N human tau (eGFP-tau) to visualize tau localization and subjected to treatment with α S monomers (α S mono), α S pre-formed fibrils (α S PFF), or phosphate-buffered saline (PBS) control. Neurons were treated with PBS, α S mono, or α S PFFs and underwent live-cell imaging 24 hours (hrs) post-treatment. Scale bar: 10 μ m. **b**. Quantification of spines containing tau as measured via DsRed and eGFP-tau co-localization. $F_{(4,70)} = 40.30$, $p < 0.0001$, by one-way ANOVA with Tukey's posthoc analysis. Cells/culture: $n_{\text{PBS}} = 21/4$, $n_{0.10\text{-PFF}} = 31/4$, $n_{4.00\text{-PFF}} = 16/4$, $n_{0.10\text{-MONO}} = 5/2$, $n_{4.00\text{-MONO}} = 5/2$. **c**. Quantification of spine density as measured via spines per pixel length of dendrite. $F_{(4,65)} = 10.87$, $p < 0.0001$, by one-way ANOVA with Tukey's posthoc analysis. Cells/culture: $n_{\text{PBS}} = 19/4$, $n_{0.10\text{-PFF}} = 22/4$, $n_{4.00\text{-PFF}} = 13/4$, $n_{0.10\text{-MONO}} = 8/2$, $n_{4.00\text{-MONO}} = 8/2$. One-way ANOVA: ** $p < 0.01$ and **** $p < 0.0001$. Error bars represent mean \pm S.E.M.

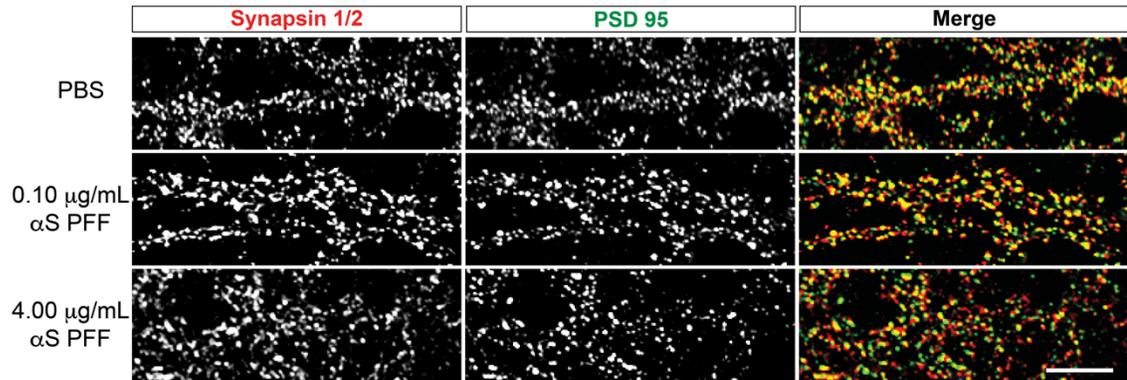


Figure 3.3 – Long-term $\alpha\text{S PFF}$ exposure produces dose-dependent synapse loss. Confocal images of rat primary hippocampal neurons treated with $\alpha\text{S PFFs}$ or PBS control, fixed 72 hrs post-treatment, and immunohistochemically stained for pre (Synapsin 1/2) and postsynaptic (PSD95) markers. Scale bar: 50 μm .

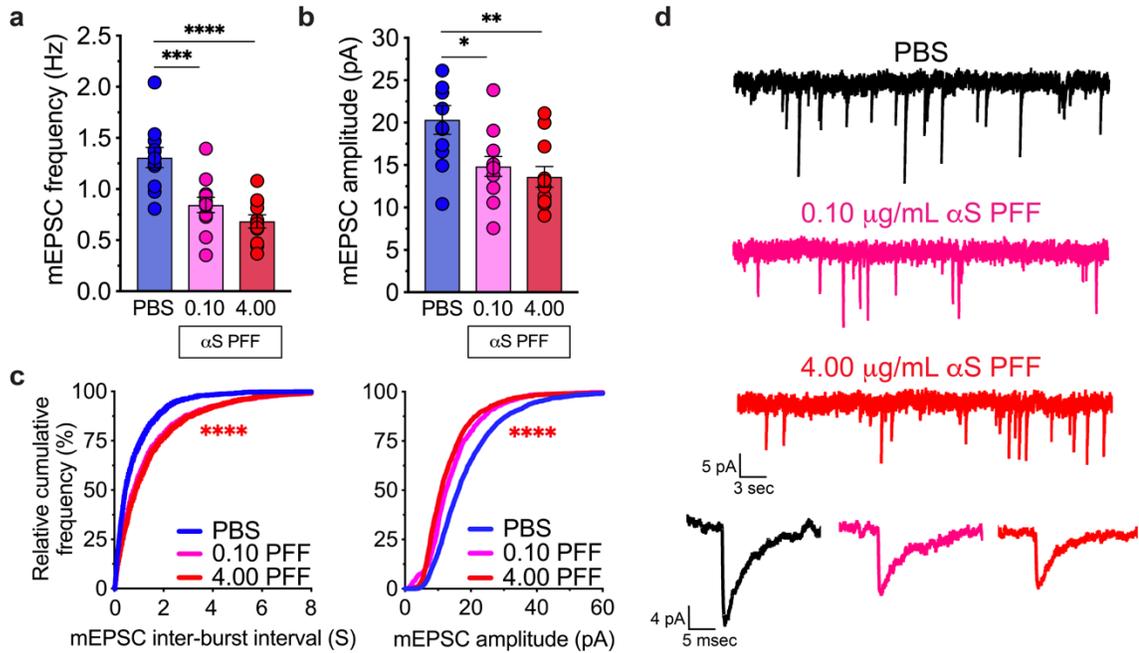


Figure 3.4 – Acute exposure to high concentrations of α S PFFs induces pre and postsynaptic dysfunction. Average mini excitatory post-synaptic current (mEPSC) frequency (**a**) and amplitude (**b**) from *in vitro* recordings utilizing rat primary hippocampal neuronal cultures. Each point represents average mEPSC frequency or amplitude from recording of a single neuron, with neurons coming from multiple litters (cultures). **a.** mEPSC frequency: $F_{(2,31)} = 15.60$, $p < 0.0001$, by one-way ANOVA with Tukey's posthoc analysis. **b.** mEPSC amplitude: $F_{(2,31)} = 6.701$, $p = 0.0038$, by one-way ANOVA with Tukey's posthoc analysis. **c.** Relative cumulative distribution frequency (CDF) of whole-cell mEPSC inter-burst interval (frequency, left panel) and amplitudes (right panel) from recordings of cultured neurons. mEPSC inter-burst interval CDF: Kolmogorov-Smirnov (K-S) test, $D = 0.9096$, $p < 0.0001$. mEPSC amplitude CDF: K-S test, $D = 0.2186$, $p < 0.0001$. **d.** Representative traces. Cell/cultures: $n_{\text{PBS}} = 11/4$, $n_{0.10\text{-PFF}} = 13/2$, $n_{4.00\text{-PFF}} = 11/2$. t and K-S tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars represent mean \pm S.E.M.

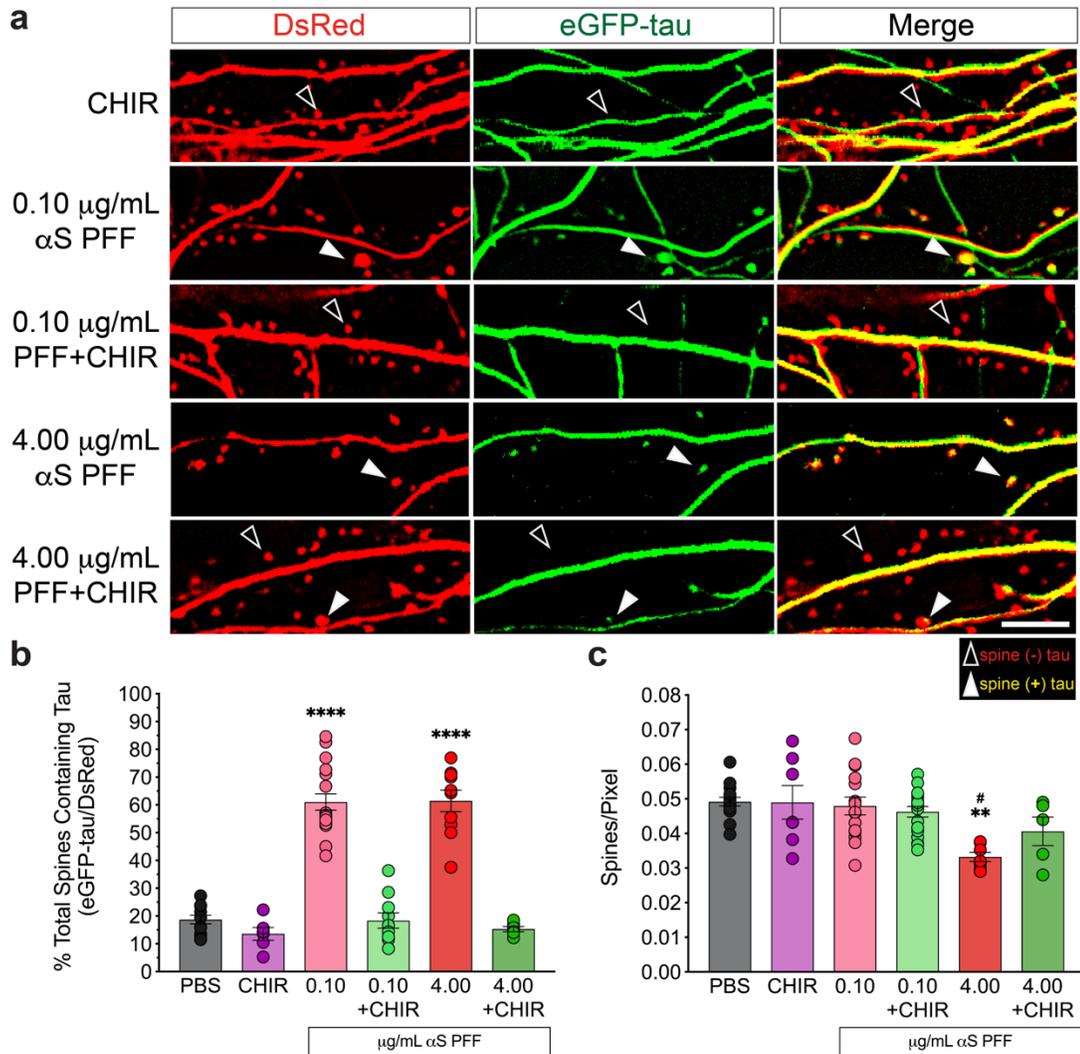


Figure 3.5 – α S PFF-mediated tau misrouting is GSK3 β -dependent. **a**. Cultured rat primary hippocampal neurons were co-transfected with plasmid expressing DsRed to visualize neuronal architecture or eGFP-tagged to 4R0N human tau (eGFP-tau) to visualize tau localization, pre-treated with the GSK3 β antagonist 500 nM CHIR 99021 (CHIR) for 12 hrs, and then exposed to α S PFFs. Neurons were then subject to live-cell imaging 24 hrs post-treatment. Scale bar: 10 μ m. **b**. Quantification of spines containing tau as measured via DsRed and eGFP-tau co-localization. Two-way ANOVA with Sidak's posthoc analysis revealed a significant effect of α S PFF exposure ($F_{(2,58)} = 35.05$, $p < 0.0001$), significant effect of CHIR treatment ($F_{(1,58)} = 147.50$, $p < 0.0001$), and significant α S PFF*CHIR interaction ($F_{(2,58)} = 24.91$, $p < 0.0001$) on tau mislocalization. Cells/culture: $n_{\text{PBS}} = 14/2$, $n_{\text{CHIR}} = 6/2$, $n_{0.10\text{-PFF}} = 17/3$, $n_{0.10\text{-PFF-CHIR}} = 10/3$, $n_{4.00\text{-PFF}} = 10/3$, $n_{4.00\text{-PFF-CHIR}} = 7/3$. **c**. Quantification of spine density as measured via spines per pixel length of dendrite. Two-way ANOVA with Sidak's posthoc analysis revealed a significant effect of α S PFF exposure ($F_{(2,61)} = 9.366$, $p = 0.0003$), no significant effect of CHIR treatment ($F_{(1,61)} = 0.7176$, $p = 0.4003$), and no significant α S PFF*CHIR interaction ($F_{(2,61)} = 1.420$, $p = 0.2496$) on spine density. Cells/culture: $n_{\text{PBS}} = 14/2$, $n_{\text{CHIR}} = 7/2$, $n_{0.10\text{-PFF}} = 14/3$, $n_{0.10\text{-PFF-CHIR}} = 18/3$, $n_{4.00\text{-PFF}} = 8/3$, $n_{4.00\text{-PFF-CHIR}} = 7/3$. Two-way ANOVA: ** $p < 0.01$, **** $p < 0.0001$, and # denotes Sidak posthoc analysis $p < 0.001$ between PBS and 4.00 μ g/mL α S PFF condition spine densities (**c**). Error bars represent mean \pm S.E.M.

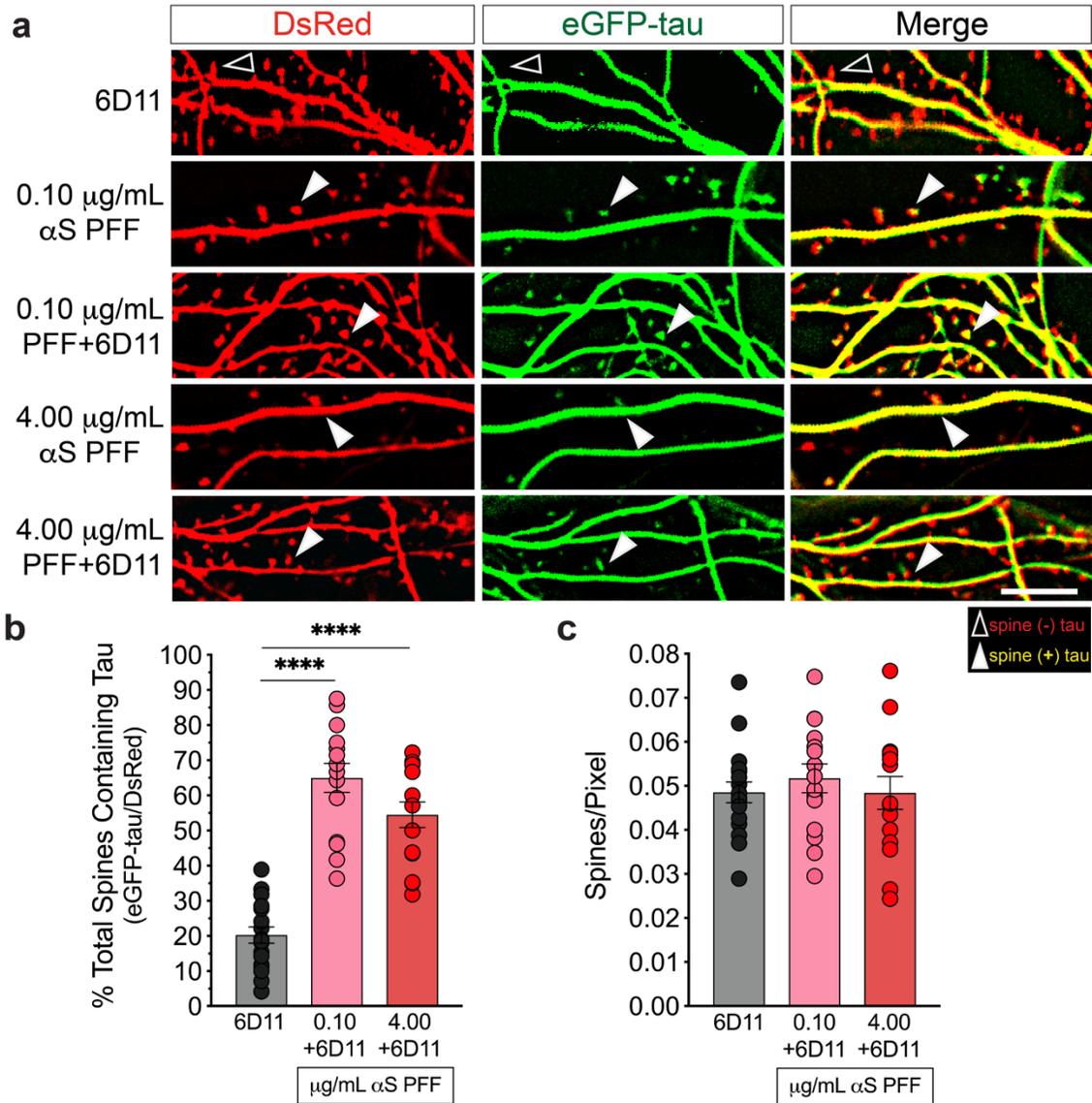


Figure 3.6 – Differential requirements of PrP^C on αS-PFF induced neuronal changes. **a.** Cultured mouse primary hippocampal neurons were co-transfected with plasmid expressing DsRed to visualize neuronal architecture or eGFP-tagged to 4R0N human tau (eGFP-tau) to visualize tau localization, pre-treated with 100 nM PrP^C inhibitor 6D11 for 4 hrs, and then exposed to αS PFFs. Neurons then underwent live-cell imaging 24 hrs post-treatment. Scale bar: 10 μm. **b.** Quantification of spines containing tau as measured via DsRed and eGFP-tau co-localization. $F_{(2,45)} = 52.11$, $p < 0.0001$, by one-way ANOVA with Tukey's posthoc analysis. Cells/culture: $n_{6D11} = 18/2$, $n_{0.10-6D11} = 15/2$, $n_{4.00-6D11} = 15/2$. **c.** Quantification of spine density as measured via spines per pixel length of dendrite. $F_{(2,45)} = 0.3569$, $p = 0.7018$, by one-way ANOVA with Tukey's posthoc analysis. Cells/culture: $n_{6D11} = 18/2$, $n_{0.10-6D11} = 15/2$, $n_{4.00-6D11} = 15/2$. One-way ANOVA: **** $p < 0.0001$. Error bars represent mean \pm S.E.M.

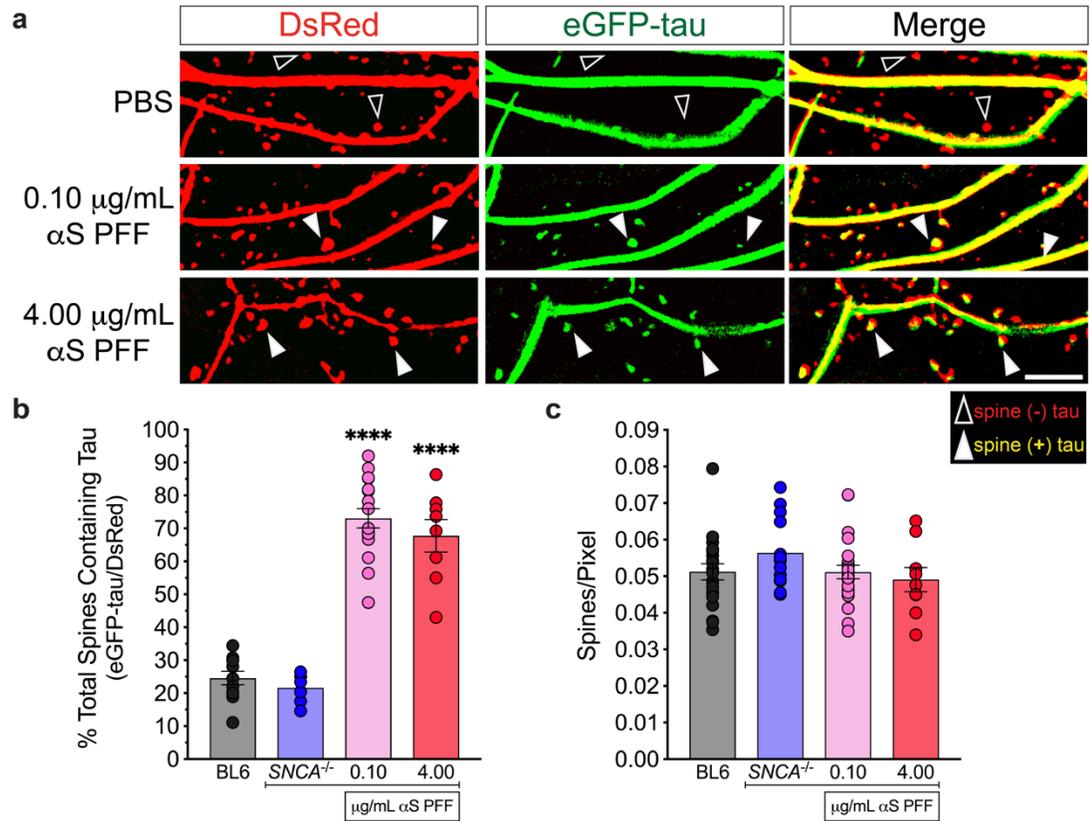


Figure 3.7 – Dose-dependent, αS PFF-induced spine loss requires endogenous αS . **a.** Cultured mouse primary hippocampal neurons from C57BL6/J (BL6) or $SNCA^{-/-}$ mice that lack endogenous mouse αS expression, were co-transfected with plasmid expressing DsRed to visualize neuronal architecture or eGFP-tagged to 4R0N human tau (eGFP-tau) to visualize tau localization and exposed to αS PFFs. Neurons then underwent live-cell imaging 24 hrs post-treatment. Scale bar: 10 μm . **b.** Quantification of spines containing tau as measured via DsRed and eGFP-tau co-localization. $F_{(3,40)} = 78.77$, $p < 0.0001$, by one-way ANOVA with Tukey's posthoc analysis. Cells/culture: $n_{\text{BL6}} = 20/2$, $n_{\text{SNCA}^{-/-}} = 13/2$, $n_{0.10\text{-PFF}} = 24/2$, $n_{4.00\text{-PFF}} = 9/2$. **c.** Quantification of spine density as measured via spines per pixel length of dendrite. $F_{(3,62)} = 1.288$, $p = 0.2865$, by one-way ANOVA with Tukey's posthoc analysis. Line underneath bar graphs refers to cultures of mouse $SNCA^{-/-}$ neurons. Cells/culture: $n_{\text{BL6}} = 11/2$, $n_{\text{SNCA}^{-/-}} = 8/2$, $n_{0.10\text{-PFF}} = 16/2$, $n_{4.00\text{-PFF}} = 8/2$. One-way ANOVA: **** $p < 0.0001$. Error bars represent mean \pm S.E.M. Contributions: HMM.

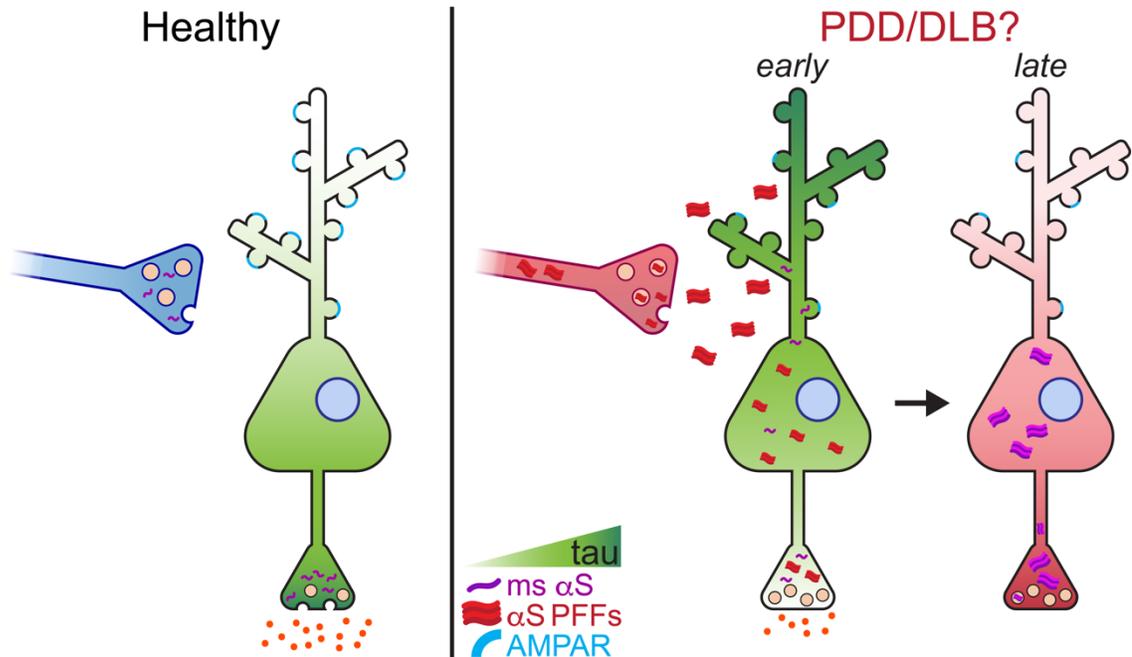


Figure 3.8 – Proposed model of disease progression and α S PFF-mediated synaptic and neuronal deficits. Under physiology “healthy” conditions, α S aggregates are absent, along with synaptic and memory deficits. However, in α -synucleinopathies associated with prominent impairment such as PDD and DLB, hippocampal Lewy pathology may be suggestive of underlying synaptic dysfunction. Pathological α S species associated with LNs in CA2 projections may be actively or passively released from presynaptic terminals through a variety of mechanisms which are currently not yet known but potentially include direct penetration through presynaptic plasma membrane, tunneling nanotubes, or release in exosome or secretory vesicles. Transmission of pathological α S from CA2 to CA1 pyramidal neurons likely leads to acute “early” and chronic “late” neuronal responses, dictated by stage and timing of disease progression. Early responses, prior to overt inclusion formation and α S aggregation, may include pre and postsynaptic deficits that impair synaptic plasticity and cognitive function. These may be a product of direct actions of pathogenic α S from CA2 neurites but can also via pathogenic α S recruiting endogenous α S and other protein intermediates like tau. The late stage is suggestive of more global compromised neuronal function, with the formation of new inclusions comprised of endogenous α S appearing and further exacerbating neuronal deficits. PDD: Parkinson’s disease dementia. DLB: dementia with Lewy bodies. ms α S: endogenous mouse α S. α S PFF: h α S^{WT} pre-formed fibrils. AMPAR: AMPA receptor.

CONCLUSIONS

Findings

The overarching objective of this thesis was to examine the mechanisms underlying neuronal dysfunction and cognitive decline in the α -synucleinopathies, PDD and DLB in particular, through several approaches. In the first arm, studies utilizing the TgA53T mouse model of α -synucleinopathy demonstrate that $h\alpha S^{A53T}$ mediates progressive synaptic and memory deficits in a tau-dependent manner. One mechanism contributing to memory deficits revealed through this work occurs through postsynaptic dysfunction via reduced AMPAR expression and depressed glutamatergic signaling. $h\alpha S^{A53T}$ -driven, tau-dependent, remodeling of hippocampal networks observed here suggests aberrant network hyperactivity also represents a driving factor of cognitive dysfunction in TgA53T mice. Findings from the longitudinal approach used to investigate cognitive decline in TgA53T mice suggests that memory deficits in these animals may be a consequence of disruptions in the balance between excitatory and inhibitory tone particularly within the limbic system and hippocampus produced via AMPAR deficits. The second arm of this thesis characterizes synaptic and neuronal deficits utilizing an *in vitro* model of α -synucleinopathies that serves to recapitulate key processes in sporadic cases of PDD and DLB. Through this approach, it became evident that several features of α S-mediated cognitive decline in TgA53T mice may also be relevant in this model. For example, pathogenic fibrillar α S-induced tau missorting to dendritic spines and AMPAR deficits, independent of overt neurodegeneration, may represent a common pathway underlying synaptic and cognitive dysfunction in both familial and sporadic cases of PDD and DLB.

Two larger, more global, conclusions can be derived from this study which also may be applicable to other neurological disorders. The first conclusion highlights the importance of postsynaptic dysfunction as a critical mechanism driving cognitive deficits.

The second conclusion proposes that different components of neuronal dysfunction and disease-associated phenotypes likely occur through differing mechanisms, as opposed to a single common pathway, such as memory deficits versus locomotor deficits in the α -synucleinopathies. It is likely that a variety of factors, including neuronal subtype and neuroanatomical location, contribute to these findings, but further investigation is warranted to better define these differing components of disease pathophysiology.

Discussion

It is clear that addressing neurological disorders and cognitive dysfunction represents a global health priority. What is less clear, unfortunately, is how to do so. Through this thesis work and in relation to other currently published studies, approaching neurodegenerative diseases through a network-level lens may yield interesting and exciting results. Concepts relating to neuron dysfunction, memory deficits, and disease pathogenesis in AD may be applicable to other diseases like the α -synucleinopathies. From a neuropathological perspective similar to AD, PD also targets specific and progresses also functionally connected networks (Braak and Del Tredici, 2016; Braak et al., 2003). In addition, altered neuronal network function and hyperactivity are implicated as drivers of cognitive decline in AD (Chin et al., 2005; Martinez-Losa et al., 2018; Palop et al., 2007; Sanchez et al., 2012), a mechanism gaining increasing traction in PD as presented in thesis and by others (Morris et al., 2015). This thesis identifies two pathological species of α S, $h\alpha S^{A53T}$ and α S PFFs, as mediators of postsynaptic dysfunction and synaptic depression at excitatory, glutamatergic synapses. Aberrant excitatory activity is also observed in aged TgA53T mice, a potential reflection of a more global network imbalance in excitatory and inhibitory tone due to dysfunction at GABAergic synapses in addition to glutamatergic. As such, the relationship between AMPAR deficits,

synaptic depression, network disturbances, and epileptiform changes may represent a common mechanism underlying memory dysfunction in PDD and DLB, although the exact mechanism linking these observations are not yet known. In support of this, cultured neurons exposed α S PFFs display altered network connectivity and reductions in excitatory tone specifically to AMPARs (Volpicelli-Daley et al., 2011), perhaps representing the first step towards network-level dysfunction in this model and possibly sporadic PDD and DLB cases. Indeed, epileptic activity, seizures, and myoclonus are common features in PD and DLB, supporting network hyperactivity and dysfunction as a clinically-relevant disease process (Beagle et al., 2017; Gruntz et al., 2018; Morris et al., 2015; Son et al., 2016).

Surprisingly, insight into network level changes in neurodegenerative diseases may also be found in pediatric conditions. Early infantile epileptic encephalopathy (EIEE) is a neurological disorder that has the unfortunate distinction of being one of the earliest and most severe forms of epilepsy also associated with cognitive disability. Mutations in the *MUNC18-1* gene encoding for the Munc18-1 (Munc) protein, are linked to EIEE (Saito et al., 2008; Stamberger et al., 2016). EIEE-associated Munc mutations lead to reduced exocytosis and deficits in neurotransmission, highlighting a role of Munc in synaptic function (Martin et al., 2014; Shen et al., 2015). The synaptic function of Munc has recently been expanded to include chaperone for α S (Chai et al., 2016). Interestingly, this chaperone function is impaired in Munc harboring EIEE-associated mutations, leading to intraneuronal α S aggregate formation (Chai et al., 2016), highlighting a role for pathological species of α S in driving neuronal dysfunction that ultimately produces seizure activity. Although Munc mutations have not yet been implicated in PD, it could be hypothesized that *SNCA* mutations, such as the A53T missense mutation, would also

impair the ability for Munc to function as a chaperone for α S, resulting in synaptic dysfunction and an imbalance in neurotransmission leading to network hyperexcitability.

From a more regional perspective, the studies using α S PFFs further support the hypothesis trans-synaptic spread of pathologic α S in the hippocampus onto CA1 pyramidal neurons in DLB may represent one mechanism leading to memory impairment (Adamowicz et al., 2017). This progression is reminiscent of the spread of pathological tau in mice also along functionally connected hippocampal circuits, from the entorhinal cortex to CA1 (Liu et al., 2012). Interestingly, propagation of tau and tau pathology is enhanced by increasing neuronal activity both *in vitro* and *in vivo* (Wu et al., 2016). However, similar to the observations of synaptic and network-level changes in TgA53T mice reported here, tau-mediated circuit dysfunction is independent of tau pathology in the rTg4510 tauopathy mouse model (Busche et al., 2019). As such, this common thread between neuronal pathology, neuronal activity, and circuit connectivity implicates network hyperexcitability not only as a driver as memory deficits in PDD and DLB, but also in accelerating disease progression. Such aberrant excitatory activity within hippocampal circuitry may additionally serve to exacerbate cognitive decline in a disease-associated feedforward loop of sorts by increasing the trans-synaptic spreading of pathological α S species to other anatomically- and functionally-connected areas of the hippocampus and cortex required for learning and memory.

While PDD and DLB are neuropathologically classified as α -synucleinopathies, disease pathophysiology extends beyond α S. This thesis introduces tau as a direct mediator of pathological α S-driven synaptic and cognitive deficits. The apparent synergy between α S and tau stems from postmortem studies identifying neurofibrillary tau tangles in individuals with genetic forms of PD (Duda et al., 2002; Kotzbauer, 2004) and that the extent of tau pathology correlates inversely with cognitive status in PDD and DLB (Irwin

et al., 2013). Overexpression of h α S^{WT} in Tg mouse models of AD has been shown to accelerate cognitive decline (Clinton et al., 2010; Khan et al., 2018; Larson et al., 2012, 2017). Conversely, removal of endogenous α S in hAPP-J20 mice rescues cognitive deficits while simultaneously increasing plaque burden (Khan et al., 2018). In addition, while α S and tau can induce each other's fibrilization *in vitro* (Giasson et al., 2003), soluble tau oligomers have been shown to enhance toxicity of α S oligomers and mediate h α S^{A53T}-mediated biochemical and behavioral changes in TgA53T mice (Castillo-Carranza et al., 2018; Gerson et al., 2018). Although α S-rich Lewy pathology and tau tangles are pathological hallmarks that effectively serve as biomarkers for cognitive status, our findings in TgA53T mice and neurons exposed to α S PFFs, along with these studies, provide further support for neuronal and memory dysfunction occurring independently of overt inclusions, with soluble species of α S and tau, such as oligomers, at the helm.

These studies directly implicate tau as a critical mediator of pathologic α S-driven synaptic and behavioral deficits in TgA53T mice, reminiscent of the role pathological tau plays in several hAPP mouse models (Ittner et al., 2010; Roberson et al., 2007). Though the physiologic role of tau is not fully established, it can be hypothesized that tau adopts pathologic roles that represent a gain of function in PDD, DLB, and AD due to its ability to undergo phosphorylation and mislocalization to postsynaptic sites to modulate NMDAR and AMPAR activity. From a therapeutic perspective, however, attempting to reduce tau expression to treat dementia in the α -synucleinopathies and AD may not represent the ideal target. Tau deficiency in mice is associated with altered microtubule organization in certain axons (Harada et al., 1994) and subtle alterations in behavior (Ikegami et al., 2000). More recently, genetic ablation of tau has been shown to impair insulin signaling within the hippocampus (Marciniak et al., 2017). Insulin resistance is a cardinal feature of neurons in AD (de la Monte, 2014), suggesting that tau may contribute to synaptic and

hippocampal dysfunction in neurodegenerative diseases through dual gain and loss of function mechanisms. To that end, it could be hypothesized that tau function in health and disease is regulated by post-translational modifications such as phosphorylation or acetylation. In addition to increasing the risk for PDD and DLB (Goris et al., 2007; Orme et al., 2018), the *MAPT* H1 haplotype is also associated with glucose intolerance (Prokopenko et al., 2014; Saxena et al., 2010). Significantly, exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist has neuroprotective effects in PD models both *in vitro* and *in vivo* (Li et al., 2009), with recent clinical trials demonstrating its clinical benefit in partially alleviating motor and cognitive deficits in PD (Athauda et al., 2017, 2019; Aviles-Olmos et al., 2014). Taken together, it is possible that the tau-dependent deficits in AMPAR signaling and cognitive function in TgA53T mice, and potentially α S PFF-treated neurons, may represent downstream consequences of α S-initiated, tau-mediated, deficits in neuronal metabolism, a hypothesis for future studies to test.

A central hypothesis tested in this thesis is the connection between pathologic α S, postsynaptic dysfunction and cognitive deficits. $h\alpha$ S overexpression ($h\alpha$ S^{WT}, $h\alpha$ S^{A30P}, or $h\alpha$ S^{A53T}) leads to presynaptic deficits, yet only $h\alpha$ S^{A53T}-expressing neurons also display reductions in mEPSC amplitude and LTP (Teravskis et al., 2018). Moreover, TgA53T mice, but not TgWT or TgA30P display deficits in hippocampal-dependent spatial learning and memory at 12 months. Moreover, these deficits in TgA53T mice are tau-dependent and a product glutamatergic dysfunction rather than synapse or neuronal loss. But what are the exact mechanisms behind this relationship? The postsynaptic protein neurogranin (Ng) is involved in synaptic plasticity (Zhong and Gerges, 2012; Zhong et al., 2009), is emerging as a promising diagnostic and prognostic biomarker in cognitive impairment and AD, and may represent a potential link between α S, tau, and postsynaptic deficits. Cerebrospinal fluid (CSF) from AD patients contains increased Ng levels (Kester et al.,

2015; Tarawneh et al., 2016). Correspondingly, Ng is decreased in brains of AD patients (Kvartsberg et al., 2019). Increased levels of CSF Ng are also predictive for progression from MCI to AD (Kester et al., 2015), and can also correlate with memory performance independent of AD-related biomarker status (Casaletto et al., 2017), supporting the specific relationship between postsynaptic integrity and memory. In detail, Ng is a critical protein for synaptic strength, LTP, and spatial learning and memory (Pak et al., 2000; Zhong et al., 2009), likely via the modulation and regulation of calmodulin and CaMKII (Hoffman et al., 2014; Pak et al., 2000; Zhong and Gerges, 2012). Protein kinase C (PKC) mediates Ng phosphorylation and activation (Díez-Guerra, 2010), and h α S overexpression has been demonstrated to reduce PKC levels and activity *in vitro* (Jin et al., 2011; Ostrerova et al., 1999), highlighting potential avenues for α S-mediated postsynaptic deficits. Although the position for tau within this pathway is not yet apparent, the significant correlation of Ng levels with total tau and pathological phosphorylated tau levels within the CSF of cognitively impaired and AD patients (Kester et al., 2015) supports a possible mechanistic interaction between these proteins and α S.

With a focus on investigating postsynaptic dysfunction in α S-dependent cognitive impairment, an unexpected finding was the requirement of tau for h α S^{A53T}-mediated suppression of presynaptic vesicle release. Tau is distributed through the axons and soma of mature neurons under physiologic conditions. Tau missorting under pathological states has classically been examined at the postsynaptic site (Hoover et al., 2010; Ittner et al., 2010; Teravskis et al., 2018). However, tau is also observed in synaptic terminals of AD brains (Fein et al., 2008; Sokolow et al., 2015) and synaptosomes isolated from Tg mice overexpressing human mutant P301L tau (hTau^{P301L}) (Harris et al., 2012; Sahara et al., 2014), suggesting that presynaptic tau may precipitate presynaptic dysfunction. Recently, hTau^{P301L} has been demonstrated localizing to presynaptic terminals and colocalizing with

CSP α (Zhou et al., 2017), a protein that coordinates with α S in SNARE complex assembly (Chandra et al., 2005), providing a potential mechanistic link between these two proteins. Zhou and colleagues (2017) further demonstrate that this presynaptic tau binds synaptic vesicles and reduces synaptic transmission by reducing vesicle recycling and vesicle pool mobilization, reminiscent of mechanisms underlying h α S^{WT}-mediated presynaptic depression (Nemani et al., 2010; Scott and Roy, 2012). Surprisingly, hTau^{P301L} and h α S^{WT} are both capable of actin polymerization and F-actin stabilization (Ordonez et al., 2018; Zhou et al., 2017), which can lead to cross-linking and immobilization of synaptic vesicles. Taken together, pathologic species of tau and α S appear to produce presynaptic deficits through common protein mediators and mechanisms. However, more work is essential to test the hypothesis that these pathological changes reflect converging, rather than parallel, pathways, of α S and tau, especially in different model systems given that h α S^{A53T}-mediated pre and postsynaptic and memory deficits are dependent on endogenous WT tau species and levels.

Ultimately, while hTau^{P301L} overexpression in mice causes presynaptic tau pathology and presynaptic electrophysiological deficits, these are not sufficient for the manifestation of cognitive deficits (Harris et al., 2012; Polydoro et al., 2014), supporting the hypothesis and findings in this thesis that postsynaptic deficits as essential drivers of cognitive dysfunction in models of α -synucleinopathy, and may apply to dementia in other neurodegenerative diseases. Still, the investigation of tau in presynaptic biology is essential to determine the mechanisms underlying the transmission of pathological species of tau and α S between neurons and brain regions, particularly with implications for the development of cognitive deficits given the similar pathological progressions within hippocampal circuitry in AD, PD, and DLB (Adamowicz et al., 2017; Braak and Del Tredici, 2016; Vasili et al., 2019).

Future Directions

The major findings of this thesis directly implicate tau as a mediator of α S-induced hippocampal synaptic dysfunction as a driver of cognitive decline. Still, future studies are essential to expand upon this mechanism and identify clinically feasible therapeutic targets. In particular, it is of importance to better define the connection between postsynaptic depression in excitatory glutamatergic synapses within the hippocampus, chronic aberrant excitatory neuronal activity, and remodeling of hippocampal circuitry. In these studies, h α S^{A53T}-mediated synaptic depression is examined exclusively in the context of excitatory synapses within the hippocampus. Furthermore, in our model, we hypothesize that the excitation-inhibition balance within hippocampal networks is disrupted and tilted in favor of chronic hyperexcitability and seizure-like changes, likely driving the hippocampal remodeling in TgA53T mice. It is possible that this hyperactive state may be produced due to inhibitory, GABAergic neurons, demonstrating an increased level synaptic depression as compared to their glutamatergic counterparts. However, it is also possible that this network-level phenotype may be a product of decreased excitatory, glutamatergic, tone on inhibitory interneurons within the hippocampus and CA1 outputs such as the subiculum and entorhinal cortex. While the hAPP-J20 mouse model of AD also displays hippocampal markers of aberrant network excitability, this pathological imbalance between GABAergic and glutamatergic signaling is shifted towards increased inhibitory activity (Palop et al., 2007; Sun et al., 2009). However, if these changes are driving epileptiform activity or compensatory attempts to suppress this activity remain unknown. For example, more recent studies in hAPP-J20 mice have also demonstrated that impaired parvalbumin-positive inhibitory neuron function, associated with reduced inhibitory signaling, underlies network abnormalities and memory deficits in this model (Martinez-Losa et al., 2018; Verret et al., 2012). Additionally, pathologic reductions in

proteins critical for learning and memory in glutamatergic neurons within the dentate also correlate with memory deficits in these hAPP-J20 animals (Palop et al., 2003). Taken together, the neurotransmitter, cellular, and synaptic mechanisms driving network-level abnormalities and behavioral deficits in neurodegenerative diseases are not easily defined and distinguished, likely multi-factorial and overlapping in origin and timing in disease progression. As such, given that the findings presented in this thesis suggest that TgA53T mice are likely to display excitatory synaptic depression prior to the onset of hippocampal remodeling, it is critical to determine the contributions and timeframe of GABAergic dysfunction and inhibitory neural network abnormalities to h α S^{A53T}-mediated synaptic and memory dysfunction.

It is imperative to note that the hippocampal remodeling observed via immunohistochemical changes (calbindin, c-Fos, and NPY) are suggestive of responses to chronic aberrant excitatory activity and not direct measures of epileptiform network changes (Vezzani et al., 1999; You et al., 2017). To that end, a longitudinal study performing electroencephalogram (EEG) and electromyogram (EMG) analysis in awake, freely behaving TgA53T and TgA53T/mTau^{-/-} mice for evidence of epilepsy-related alterations (spike complexes, seizures, myoclonus, shifts in spectral power) is a logical next step to further defining mechanisms underlying cognitive dysfunction in PDD and DLB.

Cross-species interactions, such as overexpression of h α S^{A53T} in TgA53T mice already expressing mouse α S, may influence disease pathogenesis and findings in mice. While Tg mouse models of α -synucleinopathies fail to consistently display dopaminergic neurodegeneration (Dawson et al., 2010), overexpression of h α S in *Drosophila*, which do not express an endogenous α S homolog, leads to significant neuronal loss (Feany and Bender, 2000). Carrying this back to rodents, TgA53T mice lacking endogenous α S

expression exhibit exacerbated pathology (Cabin et al., 2005) and overexpression of h α S, via α S PFFs, in neurons lacking α S, β S, and γ S leads to increased fibrillization of h α S (Fares et al., 2016). Moreover, mouse α S inhibits h α S fibrillization *in vitro* (Rochet et al., 2000), and α S PFF-induced pathology is enhanced when species of target neuron α S expression matches that of the introduced α S PFF (Luk et al., 2016). Taken together, it is clear that investigating α S-mediated, tau-dependent cognitive decline in TgA53T studies could be advanced by doing so in mice that lack endogenous α S and mTau expression, but conversely express h α S^{A53T} and human tau (hTau). These animals would provide an ideal platform to investigate the synergy between α S and tau in mediating disease pathogenesis, as previous studies suggest (Castillo-Carranza et al., 2018; Giasson et al., 2003; Sengupta et al., 2015). The species-matching of α S and tau could provide new insight into the contributions of potential pathogenic oligomeric species and other higher order aggregates to synaptic dysfunction and cognitive deficits that may be absent in the TgA53T employed in this thesis.

Within the second arm of this thesis, the study of α S PFF exposure on synaptic dysfunction in primary hippocampal neurons is ripe with potential avenues of future investigation. Given the hypothesis that α S PFF-induced tau missorting to dendritic spines precipitates AMPAR deficits and postsynaptic dysfunction, it is of importance to examine if tau is indeed required for pre and postsynaptic deficits as measured via mEPSCs. The initial studies in *SNCA*^{-/-} neurons demonstrating endogenous α S is not required for tau missorting but is required for α S PFF-induced synaptotoxicity at high concentrations, would benefit from electrophysiological studies and mESPC analysis as this would provide measures of the roles α S PFFs and endogenous α S play in early synaptic dysfunction, prior to the onset of overt inclusion formation. Along these lines, establishing the time course for neuronal responses to 0.10 μ g/mL α S PFF treatment, similar to what has been

established for 4.00 $\mu\text{g}/\text{mL}$ (Volpicelli-Daley et al., 2014), would allow for a greater understanding of the role pathogenic αS species and aggregates play in driving synaptic and neuronal deficits. Although 0.10 $\mu\text{g}/\text{mL}$ αS PFF is a concentration above the threshold needed for the conversion of monomers to fibrils (Afitska et al., 2019), it could be hypothesized that there would be a greater lag time between αS PFF application and appearance of inclusions for the 0.10 $\mu\text{g}/\text{mL}$ concentration when compared to 4.00 $\mu\text{g}/\text{mL}$. This would provide for a greater window to examine αS PFF-mediated neuronal dysfunction prior to the onset of overt neuropathology.

Although αS PFFs are the species of αS being applied, the exact pathogenic species responsible for structural and functional deficits in culture is unknown. It is therefore feasible that intermediates from αS PFF catabolism within neurons or pre-fibrillar aggregates composed of endogenous αS may be the culprit. As such, comparing the species of αS present within neurons along the established timeline for αS PFF-induced neuronal alterations would provide for insight into the kinetics behind recruitment αS PFF processing and the aggregation, oligomerization, and fibrillization of endogenous αS . Another interesting concept potentially worth pursuing is how different strains of fibrillar αS affect hippocampal neuron function. Recent studies have demonstrated differential neuronal susceptibility to αS PFFs that positively correlates with endogenous αS expression (Luna et al., 2018) and that different αS strains have differing seeding, pathogenic, and neurotropic preferences (Bousset et al., 2013; Guo et al., 2013; Peelaerts et al., 2015). In combining these two lines of investigation, the formation, spread, and cell-type specificity of different αS aggregates, such as the GCIs of MSA and the LBs of PD, is heavily dictated by intracellular environment, with little capacity for these different aggregates to cross over between different cell types (Peng et al., 2018). Building upon this, it is possible that the cortical and hippocampal αS fibrils found in PDD and DLB

patients may be different not only between those two α -synucleinopathies, but also different between the neurons in these brain regions and other regions that are affected earlier in the disease, such as the SNPC (Braak et al., 2003). Moreover, as fibrils transmit from one neuron and brain region to another, it is possible that repeated seeding of fibrils may generate slightly different strains that impart different pathogenic features, meaning that the fibrils observed in midbrain LBs are distinct from those in cortical and limbic structures. This hypothesis is consistent with the observation that midbrain and cortical LBs have different morphologies (Braak et al., 2003; Harding and Halliday, 2001). With these concepts and observations in mind, it would be interesting to examine how different α S strains impact hippocampal neuron function in culture. In addition to generating α S strains *in vitro* (e.g. fibrils and ribbons), it would be interesting to examine the potency and pathogenicity of α S aggregates produced via α S PFF induction in primary neurons cultured from different brain regions, such as the cortex and midbrain.

Ultimately, the use of several technical approaches and models to investigate the mechanisms continuing to cognitive decline in the α -synucleinopathies is critical to framing observations into a broader context and understanding of disease pathogenesis. Indeed, a major aim of this thesis was to expand upon our scientific body of knowledge in this area to better inform areas of therapeutic intervention for α S-mediated memory deficits. However, this work also provided a platform to develop a technical and experimental skillset, learn the scientific method, and become a critical, independent, and innovative scientist. Lastly, in accomplishing these aims, the findings reported here, along with their implications allow for others to continue on the investigation of the pathological processes leading to dementia in neurodegenerative diseases. Given the increasing burden of neurological disorders, compounded by an expanding and aging population, using basic

science advances to translate to and inform meaningful clinical intervention is vital for improving not just years lived, but also quality of life within those years.

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